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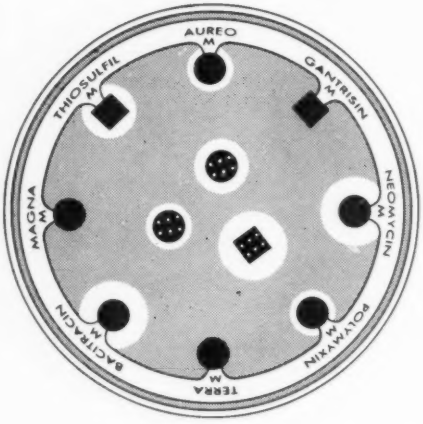

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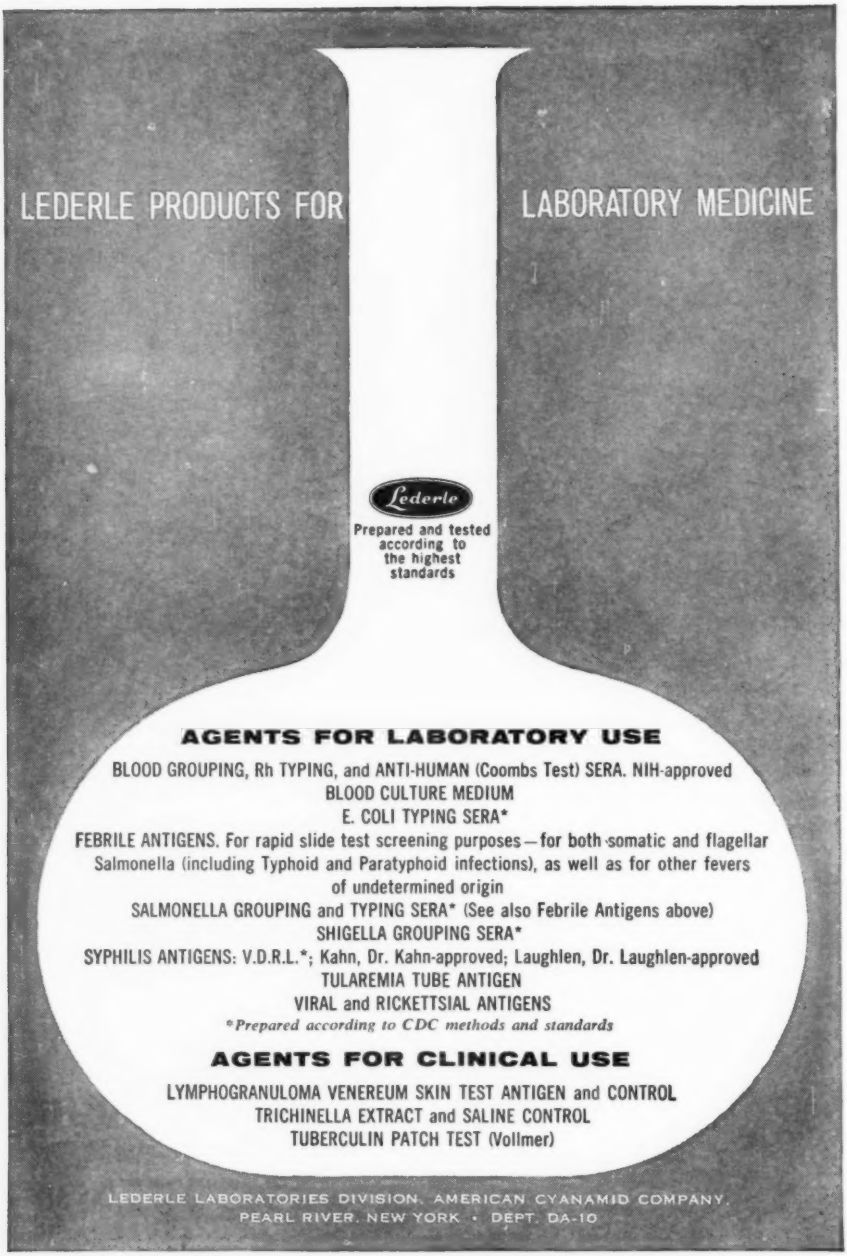
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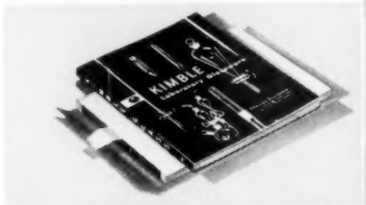

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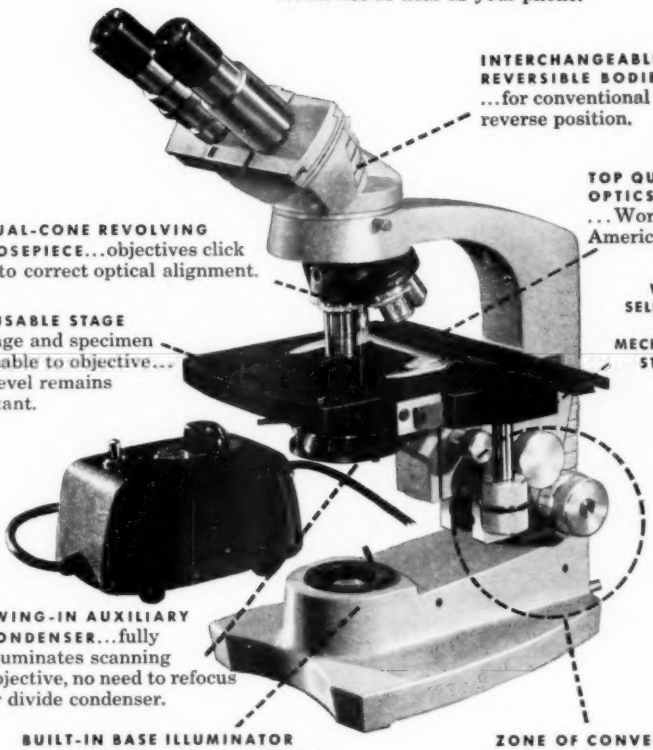

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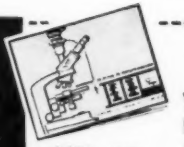

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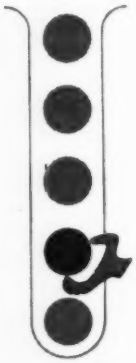

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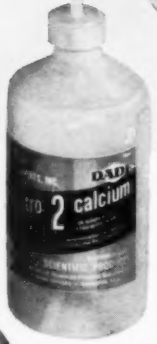

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For details, see Ferro-Ham article, "A Simple Spectrophotometric Method for the Determination of Calcium"—Technical Bulletin of the Registry of Medical Technologists, July, 1957.

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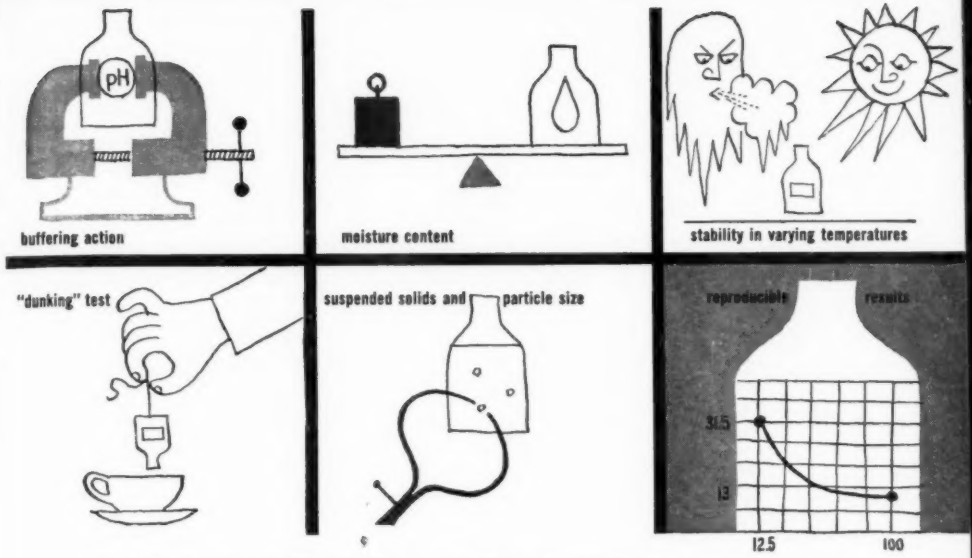

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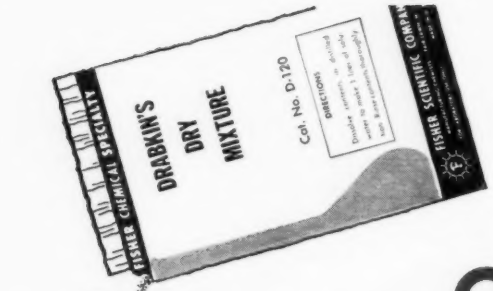

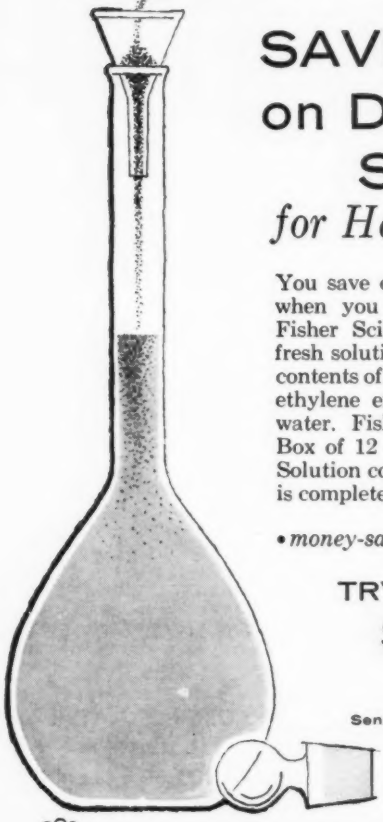

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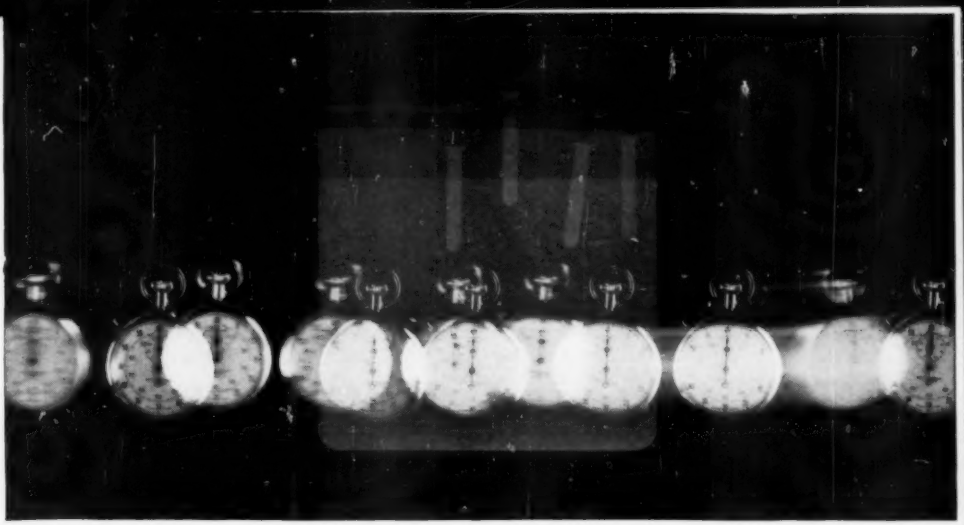

speeding up a "normal" can be dangerous

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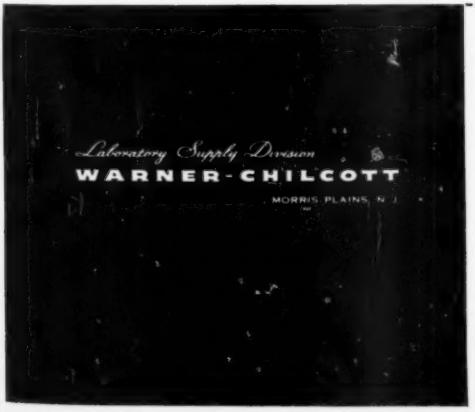

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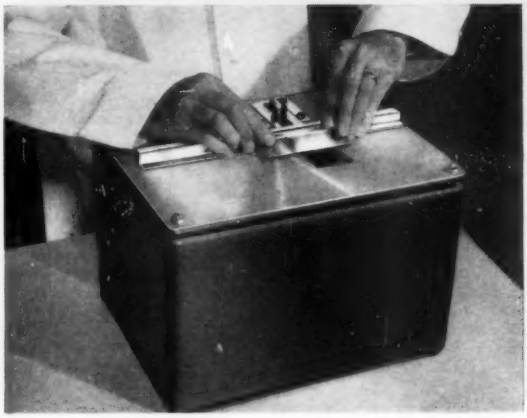

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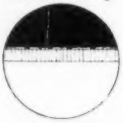

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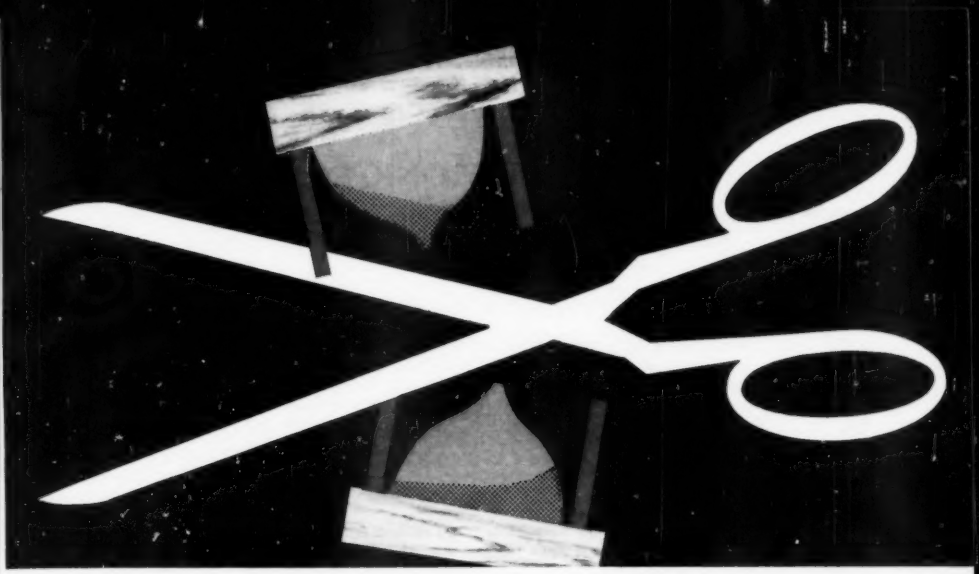

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LABORATORY DIAGNOSIS OF RABIES*

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Rabies is one of the oldest recognized infectious diseases of the animal kingdom. It was known prior to the Christian era, and has caused human fear since the dawn of civilization and recorded history. Greek mythology and stone murals of the ancient Romans refer to mad dogs and this disease. Rabies is a fatal neurotropic virus infection and is transmitted through a wound produced by a rabid animal. Man contracts the disease from some animal and the common source is the dog. From the standpoint of clinical medicine, rabies is important, not so much because of its incidence, which is low, but because of the high mortality and hopeless nature of the disease, once it has developed. The period of incubation is more variable and prolonged than any other acute infectious disease. After symptoms have appeared, the mortality in most animals approaches 100 percent. The synonym for rabies, hydrophobia, means fear of water. The characteristics of the disease involving the nervous system produce difficulty in swallowing, throat constriction, muscular contractions, respiratory spasms, cyanosis, complete paralysis and death. There are two types of rabies, furious and dumb, depending upon the symptoms shown by the animal in the early stages.

The classical work of Pasteur is the basis of our present day knowledge. He described the invisible etiological agent, the virus, as producing infection in all types of laboratory animals. Pasteur presented his experiments to the International Congress at Copenhagen, Denmark, in December 1883.[1] He had inoculated subdurally into rabbits the street virus from mad dogs of Paris. Through a series of transfers, the virus became more virulent and the incubation period was shortened. Invariably the rabbits became sick on the sixth or seventh day and died on the ninth or tenth. The virus had become 'fixed' as the virulence remained constant. The 'fixed virus' has a high degree of virulence for rabbits but has lost its potency for dogs and is avirulent for man. It has lost the power to produce disease in man but can be used to establish immunity. The Pasteur vaccine was prepared from the fixed virus of the spinal cords of the rabbits that had died with rabies.

Many modifications of the Pasteur treatment have been developed. Vaccines for humans and animals are approved by the U. S. Public

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Health Service and recommended by public health authorities for the prevention of rabies following exposure. The Semple[2a] vaccine, a killed vaccine, is most commonly used in the United States. It consists of 14 doses. Each injection is composed of 2 cc. of a four or five per cent rabbit cord suspension in physiological saline solution with 0.25 per cent phenol added as a preservative. An injection is given daily in the subcutaneous tissue of the abdominal wall. Canine vaccine, also of the Semple type, requires only one injection and the immunity produced persists for about a year.

From a diagnostic standpoint, the next important development was in 1903, when Negri,[2b] an Italian scientist, discovered small "intracytoplasmic inclusion bodies" in the cells of the nervous system. He described them as occurring in two forms,[3] one in which the inner bodies are small and very refractive, while the other form is larger, roundish, oval or irregular and less refractive. Negri believed the inclusion bodies of rabies to be one of the stages in the development of a protozoan parasite. There was no microscopic method for diagnosing rabies prior to this time. Our knowledge is limited in regard to their presence in the nerve cells, and it is this fact upon which we rely for the diagnosis of rabies at the present time.

Negri Bodies

The typical Negri bodies as found in the hippocampus consist of spherical, oval, or elongated, sharply defined eosinophilic structures. They vary in size from 2 to 10 microns or larger in diameter and are found in the cytoplasm of the neurons. Several inclusion bodies may be present in one cell. Characteristic of the Negri body are the basophilic granules of the inner structure, varying from 0.2 to 0.5 microns in diameter. In addition, single to many vacuoles may be present in the body. When one or both of these structures are not found in an eosinophilic body, the diagnosis is questionable as inclusion bodies are found in other diseases. Negri bodies are abundant in the hippocampus but usually cannot be found in as large numbers in the pyramidal cells of the cerebral cortex and the Purkinje cells of the cerebellum. The Negri body that is large and typical with a well developed inner structure of blue granules and vacuoles, as well as staining a deep red is easily recognized. In some brains, atypical bodies are seen as small eosinophilic structures, globular in nature and have been found in both the cortex and cerebellum of cats and also in normal mice that have not been inoculated with rabies. These atypical inclusion bodies cause confusion in the examination of animal brains. These refractile bodies in cats led Stovall and Pessin[13] to examine the brains of normal cows and sheep from a packing plant. Large and small homogeneous eosinophilic bodies, both intracellular and extracellular, were found in the medulla and cerebral cortex of these normal animals. The presence of these atypical inclusion bodies, makes mouse inoculation most essential for a final decision.

Occurrence of Rabies

There is a prevalent misconception that rabies is not widespread, but to the contrary, it has appeared in every state in the Union and in practically every wild animal in North and South America.[4a] Rabies

was first introduced in North America by the importation of dogs from Europe. It appeared in the vicinity of Boston and was limited to the eastern seaboard states until 1800. It spread from this focus and by 1900 was enzootic over most of the United States. All warm blooded animals are susceptible, but primarily, it is a disease of the canine (Canidae) family. In the United States, the skunk, fox and coyote are the principal wild animal vectors. The red and gray foxes are prevalent along the Appalachian Mountains from New York to Florida. The skunk and fox have been the focus of rabies in the Middle Western States. The coyote was the vector in Arizona. Wisconsin has had an epizootic of skunk and fox rabies for several years and has become an economic problem. They invade the farmyards and attack domestic animals. Whenever the wild carnivorous species become abundant in an area there is a threat that rabies may become epizootic. In South America, Central America, Mexico and Trinidad, the vampire bat is a common and principal vector. The vampire bat is the only known vector capable of transmitting rabies for several months as a "symptomless carrier."[2b] All other bats, as far as is known, transmit rabies for a very limited time and then die of the disease.

In the United States, there are workers in various localities who have reported the finding of Negri bodies in several species of bats.[5a] California has detected them in the hoary bat, *Lesiurus cinereus*, and the Mexican free-tailed bat, *Talarida mexicana*. Michigan has detected Negri bodies in the large brown bat, *Eptesicus fuscus fuscus*. This is a migratory bat observed at times during the winter in Michigan. One of these bats was found by a boy as he was returning home from school. The bat bit him and the boy took the bat home. It was placed in a cage and the bat died seven days later. The bat was sent to the laboratory and was negative for Negri bodies by means of a slide preparation, but mice inoculated were found positive for Negri bodies. Inoculation of mice with material from some normal appearing bats have developed Negri bodies. The development of "symptomless carriers" of various types of bats other than the vampire bat, has not been shown up to the present time.

Puerto Rico has reported rabies in the mongoose,[6] a small carnivorous animal, known in native India for its ability to attack and overcome the cobra and other poisonous snakes. The mongoose was imported to Puerto Rico by sugar planters for the destruction of rats which caused large economic losses. While the jackal and not the mongoose is the principal vector of rabies in India, the mongoose has become an important vector in Puerto Rico. The first reported case of rabies in the mongoose was in 1950, and the investigation showed that the dogs and cats had fought with the mongooses during a period of two weeks to two months prior to the time the dog and cats developed rabies. Due to quarantine regulations, rabies is not found in Australia or Hawaii and Great Britain has reported no cases since the disease was eradicated in 1922. Denmark, Norway and Sweden have been free of rabies for more than 100 years.

There is a popular fallacy and misconception that rabies is more prevalent during "dog days" of summer or during hot weather. Table I

shows that more animals have rabies in late winter and early spring than during the summer months.

TABLE I

Total Positive Rabies by Months in Animals in Wisconsin for a Period of 34 Years (1904-1938)

Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
79	91	99	92	52	74	72	70	76	50	47	58

The reason more animals have rabies in late winter or early spring is because stray dogs roam the countryside scavenging for food and seeking mates.

TABLE II

The Reported Cases of Rabies in Animals[5b] in the United States, 1946-1955

1946	1947	1948	1949	1950	1915	1952	1953	1954	1955
10,850	8,920	8,495	7,587	7,901	8,008	8,445	8,903	7,297	5,799

Each year many human beings are bitten, especially by dogs, and the following table shows the number of human deaths by year for the past ten years.

TABLE III

The Reported Registered Deaths from Rabies in Man[5b] in the United States for the Ten-Year Period, 1946-1955

1946	1947	1948	1949	1950	1951	1952	1953	1954	1955
34	26	24	10	18	18	24	12	13	5

In addition to these deaths from rabies there are more than 30,000 persons[7] who take a series of inoculations each year. The cost of human vaccine and livestock losses exceed $5 million per year in the United States. Approximately 90 per cent of human rabies arise from a canine source but occasionally man is infected by other animals. During a period of forty years, this laboratory has diagnosed only two cases of human rabies. One case was a boy 16 years old and the other was a boy 7 years of age. In both cases, the history was so vague that the animal or the incubation period could not be determined. This laboratory has received the following animals: dog, skunk, fox, cat, cow, horse, swine, sheep, racoon, deer, muskrat, opposum, chipmunk, woodchuck, rabbit, squirrel and rat. The dog and skunk have shown the highest percentage of Negri positive brains.

While the demonstration of inclusion bodies in the nerve cells by Negri seemed to provide a satisfactory method for the diagnosis of rabies, there still remained the necessity of the development of specific technics for its application and to take cognizance of its limitations.

Technic

As soon as possible after the death of an animal, the head is severed close to the shoulders, packed in ice and shipped in a tin container, which will prevent the leakage of fluids, to the diagnostic laboratory. Upon receipt of the head at the laboratory, the brain is removed, using rubber gloves as a precautionary measure. The skin is dissected by a midline incision and the two flaps of skin are severed between the ears, eyes and nose until the skull is completely exposed. The animal's nose is grasped by sharp bone forceps and with the aid of bone shears, the calvaria of the skull is removed. The fingers are inserted firmly beneath the brain and the whole brain is removed as one specimen. The Ammon's horn or hippocampus is the area in which Negri bodies are most prevalent. The brain is dissected by cutting in a midline between the two cerebral cortex hemispheres, with the dorsal part facing upward. Just anterior to the cerebellum, the cortex is cut through over the posterior horn of the lateral ventricle to expose the hippocampus. On the floor of the lateral ventricles is a glistening white, cylindrical cord-like structure which is the hippocampus. Cross sections are taken from the hippocampus for the preparation of smears and paraffin sections.

Smears are prepared for quick diagnosis, and paraffin sections are routine procedure in a few laboratories. The chief objections which have been made to the section technic is that it is time consuming and fraught with technical difficulties. Preparation of sections require only a few hours in addition to the time necessary for smears, the technic is not difficult and the equipment is found in most laboratories. Sections provide large numbers of large neurons for Negri body search.

Preparation of Smears

There are three methods of making smears for the quick diagnosis of rabies—the impression, rolling and spread methods. The spread method is the one which is used in this laboratory. A small section is removed from the inner portion of the Ammon's horn and placed on a glass slide. Another slide is pressed firmly on the material and against the first slide, and then the slide is pulled endwise but firmly apart on a plane parallel to their surfaces. This provides a thin film of concentrated material and a homogeneous spread of tissue.

Preparation of Tissue

At the time smears are prepared, representative specimens are selected for paraffin sections.

A. Preparation of brain tissue.
   1. 3 to 5 mm. sections are cut from the hippocampus, cerebellum and cerebral cortex.
   2. Each section is placed between two small squares of white writing paper in a Petri dish.
      The fresh brain material sticks to the paper and prevents shrinkage of the tissue in the process of fixation and dehydration.

B. Fixation and dehydration.
   1. The pieces of tissue in the Petri dish are covered immediately with acetone and left to fix for 2½ to 6 hours or overnight, depending upon the size of the sections.
   2. Pieces of paper are carefully removed before the tissue is placed in paraffin.

C. Paraffin infiltration.
1. The tissue is placed in melted paraffin (58-60° C) for 3 hours to overnight, depending upon the thickness of the tissue and the period of the working day, to thoroughly impregnate the tissue.
2. The tissue is embedded and blocked in 58-60° C. paraffin.

D. Sectioning.
1. Sections are cut 4 to 5 microns thick.
2. The paraffin ribbon of sections is floated on water at 40-45° C. to remove the wrinkles.
3. These ribbons are mounted on slides and warmed gently over a small flame to cause the paraffin section to adhere to the slide.
4. The sections are placed in an oven at 60-63° C. for 45 minutes after which time they are ready for staining.

E. Staining.

It is necessary to use a strongly selective stain for both the smears and sections, as some tissue shows only a few small Negri bodies. The smears are stained immediately after being prepared. The stain used in this laboratory for many years is the one developed by Stovall and Black.[8] Since the dry stain purchased from time to time has given inconsistent results, it has been found necessary to modify the Stovall-Black method[8] slightly by altering the pH of the two stains. (Sellers stain[15] is used to stain smears by many laboratory workers). The staining procedure which is used at the present time is as follows:

1. Xylol (2 changes) for the smears and removal of the paraffin from the sections.
2. Absolute alcohol (2 changes) to remove the xylol.
3. 95% alcohol (2 changes).
4. Distilled water—rinse thoroughly before staining.
5. Stain one minute in 1% solution of ethyl eosin in 95% alcohol to which 1 cc. of acetate-acetic acid buffer* pH 4.5 is added for each 60 cc. of solution.
6. Rinse in distilled water.
7. Stain one minute in modified Loeffler's methylene blue** to which 1 cc. of acetate acetic acid buffer pH 4.5 is added for each 60 cc. of solution.
8. Rinse in distilled water.
9. Rinse for 5 seconds in distilled water containing 5 drops of glacial acetic acid to 60 cc. of water.
10. Rinse in distilled water.
11. Rinse in 95% alcohol for differentiation—approximately 15 to 20 seconds.
12. Rinse in second 95% alcohol.
13. Absolute alcohol (2 changes) for dehydration.
14. Xylol (2 changes).
15. Mount in balsam or Permount with No. 1 cover glass.
16. Examine with oil immersion lens.

* Acetate-acetic acid buffer: Sodium acetate 25.0 grams. Acetic acid (glacial) 5.0 cc., Distilled water ad., 250 cc.

** Modified Loeffler's methylene blue: Saturated alcoholic solution methylene blue (medicinal), 15 cc.; Distilled water ad., 60 cc.

Fresh stains, that is, the addition of the distilled water and buffer to the methylene blue and the addition of the buffer to the 1% ethyl eosin, should be made every 24 hours. The 1% ethyl eosin in 95% alcohol is a saturated solution and should be mixed well before each staining.

Eosin Y[9a] commonly called yellowish water soluble eosin is known as tetrabromfluorescein, an acid dye and one of the xanthene series. This eosin is used for routine tissue staining in the hematoxylin eosin method. The yellowish eosin mixed with methyl blue was used by Negri[10] in 1903, when he discovered the peculiar red staining inclusion bodies in the central nervous system of rabid animals. In this laboratory ethyl eosin has been found superior to Eosin Y for staining Negri bodies. Ethyl eosin (alcohol soluble) is an acid dye, the ethyl ester of Eosin Y, and belongs to the xanthene series also. The large and small Negri bodies tend to stain a deeper red by decreasing the pH of the ethyl eosin. It is essential to stain the small bodies an intense red as occasionally there are only a few minute Negri bodies in the brain tissue. Methylene blue[9b] is a basic dye and belongs to the thiazin series. It is a nuclear and contrasting stain. The nucleolus of the pyramidal cells stain a dark blue with a lighter shade to the nucleus and cytoplasm of the cell. The internal granules of the red Negri bodies stain a dark blue. The pH of the methylene blue controls the depth of color in the cytoplasm of the nerve cell as well as the inner structure of blue granules of the Negri bodies.

In case the smear which is strained immediately after the dissection of the brain is positive for Negri bodies, a report is sent at once to the veterinarian, doctor or sender advising Pasteur treatment of all persons bitten. If the smear is negative, no report is sent until paraffin sections are examined the following day. After careful examination of smears and sections with oil immersion lens and they are found to be negative for Negri bodies, a report is sent at once to the sender as follows: "We have been unable to find Negri bodies in this head. It has been demonstrated that from 10-20% of animals that die of rabies will not show Negri bodies. If this animal showed symptoms of rabies, all persons who were bitten should receive the Pasteur treatment at once. In order to prove that this animal did or did not have rabies, we have injected some of the brain tissue into mice and at the end of the test (2 to 3 weeks) you will be notified of the result." All animals which are negative for Negri bodies by the smear and section technic are inoculated into each of five mice.

Mouse Test

The mouse inoculation test for rabies is a relatively simple procedure. Any of the strains of Swiss white mice may be used. The mice should range between three and six weeks old, as older mice are more difficult to inoculate because of the thickness of the calvarium.

1. Small portions of Ammon's horn, cerebral cortex, and cerebellum are pooled in a sterile mortar and thoroughly ground.
2. Sterile physiological saline in small portions is added to make a 10% suspension. The material is emulsified well, until there is a uniform suspension.
3. 100 units of penicillin per milliliter is added to the suspension to decrease the mortality of the mice, especially if the brain has under-

gone decomposition. In extreme cases, or if penicillin is not available, storage of the tissue for a few days in 100% glycerin will serve to protect the mice without destroying the virus.

4. The suspension is allowed to settle and the supernatant fluid is injected.
5. The mice are anesthetized in a covered jar by saturating fresh cotton in the bottom of the jar with ether. Mice are removed *just as they fall over*.
6. The mouse is held posterior to the ears with the left forefinger and thumb. With a cotton applicator alcoholic iodine solution is used to sterilize the area between the eye and ear for insertion of the hypodermic needle.
7. The syringe is held at an angle and the needle is inserted laterally to the median line, midway between the eye and ear.
8. 0.03 ml. of the supernatant fluid is injected subdurally.
9. If the mouse recovers before the injection is complete, it can be placed in the ether jar again for a short time. If there are signs of an excess amount of ether such as violent jerking and gasping, the mouse can be swung by the tail in a semi-circle to revive it.
10. Five mice are inoculated in this manner.
11. Daily observations are made of the mice and symptoms will develop between the sixth and tenth day if the brain was from a rabid animal. Occasionally the incubation period may be prolonged to twelve or fifteen days.
12. Symptoms are: Roughing of the fur, humping of the back, muscular tremor, incoordination, conjunctivitis and paralysis. Convulsions are often seen and they die during a seizure. Mice showing prostration are killed with chloroform and examined.
13. Removal of the mouse brain for sectioning. With the dorsal aspect of the body facing upward, the skin of the head and neck is dissected so the skull is exposed. The skull is grasped in the orbits with a small forceps and the calvarium is cut with a scissors which exposes the brain. A section about 3 mm. is cut from the brain just anterior to the cerebellum and will contain the hippocampi. The section is placed between two squares of paper in a Petri dish, fixed in acetone, and paraffin sections are prepared in the same manner as for the brain of the animal suspected of having rabies.

Some mice may die of traumatic injury during inoculation, but an experienced worker loses few mice. One mouse is killed ten days after inoculation and a positive diagnosis is frequently made at this time. The second mouse is allowed to run the full 17 days before a final report is made. The remaining mice are destroyed after 30 days if no symptoms develop.

Our mouse inoculation technic was originally based on surveys by Webster and Dawson,[11] Leach,[12] Stovall and Pessin,[13] and others who have found that 10 to 12 per cent of the brain tissue of suspected animals was positive by mouse inoculation that was negative by direct microscopic examination. Table IV will show the results obtained in this laboratory over a period of 16 years.

TABLE IV
Showing Results of Smears, Paraffin Sections and Mouse Inoculation Methods of Rabies Diagnosis

Year	Total Heads Rec'd	Net Total Heads Examined	Net Total Heads Not Examined	Heads Micro +	Heads Micro — or Unsat Mice —	Heads Micro — Mice +	Heads Micro Unsat Mice +	Heads Micro Unsat. Mice —	Percentages	
									Micro & Unsat — Mice +	Micro +
a	b	c	d	e	f	g	h	i	j	k
1940	236	227	9	102	100	24	1	7	20.0	45.5
1941	127	127	0	37	78	12	0	6	13.3	29.2
1942	71	71	0	11	57	3	0	4	5.0	15.5
1943	55	53	2	5	46	2	0	5	4.2	9.4
1944	50	50	0	9	38	3	0	4	7.3	18.0
1945	41	41	0	7	34	0	0	3	0	17.1
1946	49	49	0	2	44	3	0	4	6.4	4.1
1947	65	65	0	8	41	6	0	2	10.5	12.3
1948	62	62	0	3	41	8	0	3	13.5	4.8
1949	80	80	0	8	57	13	2	0	20.8	10.0
1950	88	84	4	9	64	11	0	2	14.7	10.7
1951	129	129	0	14	98	14	3	10	14.8	10.8
1952	232	229	3	26	176	23	4	18	13.3	11.3
1953	193	193	0	26	148	17	2	20	11.4	13.5
1954	319	318	1	43	235	36	5	15	14.9	13.5
1955	258	251	7	32	202	14	3	26	7.8	12.8
	2055	2029	26	342	1459	189	20	129	12.4*	16.6*

* Average percentage.

It will be noted the percentage of positives found by the mouse method from the total negative findings by smear and section examination is 12.4 per cent. This result is almost exactly what Leach[12] found in 1938 and Stovall and Pessin[13] found in 1942. It should be stated that the percentage of heads found positive by mouse examination is calculated on the basis of the number of negative heads (Column e subtracted from Column c), or 1,687. It will also be noted from Table IV that during this period of 16 years those heads which could not be examined by smears or sections because of decomposition, mutilation or otherwise, gave 20 positive results obtained by the mouse technic. On the other hand, the percentage positive heads by the smear and section method is based on the net total of heads examined (Column c). Furthermore, it should be stated that of the 551 positive heads obtained by the complete technic (smear, sections and mice), there were 342 obtained by smears and sections or 62 per cent, 189 obtained from brains by mouse technic which were negative by smear and section or 34.2 per cent. The 20 positives (Column h) found by mouse inoculation from brains which were unsatisfactory for smear and sections amounted to 3.6 per cent.

Wound Prophylaxis

In general the bite should be handled like any other type of wound. A bite that is inflicted by a suspected rabid animal should be washed thoroughly with warm water and soap. In case of a deep puncture wound, it should be opened surgically before washing as this will inactivate or remove the virus which is believed to remain localized at the point of introduction for an undetermined length of time. Thorough washing with 20% soft soap solution is believed to be as effective as nitric acid cauterization. Wounds were cauterized with nitric acid for many years as the result of experimental studies[2c] of Cabot (1899), Poor (1911) and Rosenau (1935), but this procedure is no longer considered advisable.

Suspected Animal

Since the suspected animal will die within ten days if rabid at the time of the bite, the animal should be corralled and observed for this period of time. If the animal is killed too soon, Negri bodies may not have developed and reliance will need to be made on the mouse inoculation method. By observing the suspected animal, time can usually be gained for making a diagnosis. If the animal is well at the end of 14 days, the animal did not have rabies at the time of the bite.

Pasteur Treatment

Persons bitten by an animal should not be given the Pasteur treatment carelessly or indiscriminately.

A. Immunization should be considered in all the following cases.
   1. Animal identified and showed definite signs of rabies.
   2. Laboratory found positive evidence of rabies.
   3. Laboratory examination negative but symptoms showed evidence of rabies in the opinion of the veterinarian.
   4. Animal not available and rabies known to be present in the community.

B. The anti-rabic treatment is not entirely harmless as partial, or at infrequent intervals, vaccine paralysis has occurred. 5,500 persons received rabies vaccine in Los Angeles City and County from 1940-1946.[14] Nine cases including one death developed severe vaccine reaction. Therefore, it is advisable to give the treatment only for the following reasons:
   1. Wounds made by teeth on the bare skin.
   2. Wounds made through torn clothing by teeth of an animal.
   3. Saliva in a raw, open, pre-existent wound that is under 24 hours old.
   4. Young children when reliable evidence cannot be obtained.
   5. Stray animal that died shortly after biting.

C. The anti-rabic treatment is not advisable when the following conditions exist:
   1. Contact by saliva on unbroken skin including the face and mouth.
   2. Contact with saliva of cuts or abrasions that are 24 hours old or protected by a serum scab.
   3. Bites, pinch or friction wounds through clothing that is not torn.
   4. Handling or petting rabid animals.
   5. Handling objects contaminated with saliva of rabid animals.
   6. Exposure limited to drinking milk of a rabid cow.
   7. Animal remaining well for one week after biting.

The diagnostic services of the laboratory has contributed a great deal to the control of rabies. It is not only a laboratory problem but a community responsibility. Rabies stands out from all other infectious diseases because once symptoms have developed the illness always ends in death. The infected animal has no respect for man or beast, state or county borders, but roams the countryside and spreads the disease from one vicinity to another.

Rabies control programs may include these broad measures:

1. Impounding and the destruction of all stray or ownerless dogs.
2. Muzzling all dogs for 90 days in an infected area.

3. The anti-rabies vaccination should be given to all dogs annually.
4. If all dogs are licensed, it tends to rid a community of stray dogs and places the responsibility upon the dog owner.

The World Health Organization Committee on Rabies is attempting to eradicate rabies in Palestine, Iran, Egypt and other Near East Countries, but "Charity begins at home." More energetic effort should be made to eradicate this fatal disease in our own country.

Summary

1. A comprehensive technic is described whereby smear, section and mouse inoculation are used to detect rabies, the Negri body forming the basis for a positive diagnosis.

2. A smear and section staining technic is described for both the brain of the suspected animal and the brain of the mouse used for the inoculation test. This stain is based on the Stovall-Black technic.

3. A summary table is included showing the incidence of rabies in Wisconsin as measured by the positive heads found in this laboratory during the last 16 years. This table shows very definitely the value of the mouse inoculation technic.

Note: Since this paper was written, six cases of rabies in bats have been diagnosed in Wisconsin from a total of 124 examined between May and September of 1957. Duck embryo vaccine is being used somewhat in this state as a prophylactic measure against rabies, alone or in combination with antirabies serum.

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APPLICATION OF SELECTIVE HISTOLOGICAL STAINS TO AIR FORCE RESEARCH*

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I. *Brain Tissue-Nerve Cell Studies*

Discussion. The study of concussion is important in the design of helmets as protective equipment for pilots of high-performance aircraft. As information is gained about the cause and effects of concussion, improvements can be made in the helmets, clothing, and cockpit or cabin design.

One of the theories regarding concussion is that it results from distortion or jerking of the head and upper spinal column in association with a blow. In support of this theory, an experiment was conducted on the mechanics of concussion in order to prove that concussion is actually caused by "stretch" or spatial distortion of the neuraxis, specifically in the atlanto-occipital region.[1]

Young adult cats were anesthetized by Dial with urethane and securely supported in the neck region. Then following a free, vertical fall, the cats were rapidly decelerated by a mechanical device.

Histopathology was used to aid in this experiment:

(a) to demonstrate brain cell damage following concussion, even though no gross changes are seen;

(b) to see in which area of the brain the most damage appears, especially significant if damage is seen in the atlanto-occipital region.

Interest was focused on the nerve cells of the brain stem and the presence or absence of Nissl bodies within the cytoplasm of these cells. They appear as granular masses, or larger bodies of linear or triangular shape[2] which give a striped or tigroid appearance to the cell, and are precipitated by the action of fixatives. This Nissl or tigroid substance may be altered in a fatigued or injured cell; "it is not precipitated in a normal manner, but appears to have undergone solution or disintegration, a reaction called chromatolysis."[3]

Technique and Results. After necropsy of the animals, the brain tissues were fixed in neutral 10% formalin or Orth's fluid, embedded in paraffin and, in the staining process, were coated with 1% celloidin solution, to keep them from tearing or falling off the slide.[4]

Lillie's Azure-Eosin stain was found very satisfactory for accenting the Nissl substance. Azure A or Azure II were equally good and Eosin Y was substituted for Eosin B. The two stains were combined with M/10 citric acid and M/5 disodium phosphate, acetone, and distilled water to give an approximate pH of 3.5-4.0. Sections were stained for 1 hour, dehydrated in acetone, cleared in an equal parts acetone-xylene mixture, and xylene, according to Lillie's technique.[5]

The Nissl bodies stain a deep blue or violet, and when they are abundant, the whole cytoplasm appears to be a mass of blue particles in linear form or clumps; while the nucleus, containing little chromatin, stains lightly or not at all. The round nucleolus stains blue and the glia background of brain tissue is light pink.

Petechial hemorrhage, always of interest in injury to the brain, was not especially evident here. In general, blood cells appear as a deep pink or coral against the pale pink glia network.

* Read before annual convention, Chicago, Ill., June 1957.

II. *Grading of Burns*

Discussion. The use of Verhoeff's stain for elastic tissue and myelin sheaths has been known for some time. Gridley modified the Mallory method,[4] and Cowdry cited a short staining time for elastic tissue, a longer timing for myelin sheaths,[6] but recently Hinshaw & Pearse applied the method in the differential staining of burned tissue.[7]

The stain was tried in our laboratory on several sections of burned skin from rats and was then chosen for a project involving the study of thermal radiation, in relation to protective clothing for Air Force flying personnel against (a) flash burns such as might occur in industrial accidents from fuel explosions and (b) effects of thermal radiation from atomic weapons.

The severity of burns is related to the time and intensity of applied thermal energy and the degree of burn can best be judged with a stain which gives a marked contrast between normal and burned tissue. The technique used by Hinshaw and Pearse with Verhoeff's and Van Gieson's stain fulfills this purpose, is easy to follow, and the resulting slides can be interpreted rapidly.

Experiment. Male Sprague-Dawley rats were clipped with surgical shears to remove hair, and 48 hours later were anesthetized with pentobarbital sodium. After a band of test fabric was fastened over the abdomen, each animal was exposed to two burns, one on each side of the lateral body wall, with each area 2.5 centimeters in diameter.[8] The source of heat was a modified N.R.D.L. Mitchell carbon arc.[9] Varied timing exposure and intensities were used to simulate atomic explosions. Representative animals were photographed in color for permanent records of the gross pathology. The animals were examined immediately after exposure and 24 hours later; and on the fourth day were sacrificed for post mortem and tissue biopsy. The burns were graded grossly into five categories, from loss of superficial layers to complete carbonization of skin. Sections were cut grossly through the center of the burned area, leaving a tag of normal tissue at one or both ends.

Technique. Representative samples from 500 burns were fixed in neutral 10% formalin, embedded in paraffin and stained with Verhoeff's, using the Hinshaw-Pearse technique. Paraffin blocks were kept in the freezing compartment of the refrigerator before cutting and were sectioned at 6 microns on the (Spencer) sliding microtome. A 1% celloidin solution was used in the staining process to keep sections intact.

Duplicate sections were cut from each burn sample and processed, using hematoxylin-eosin stain on one, and Verhoeff's-Van Gieson stain on the other, thus affording a comparison.

Results. The distinct contrast between burned and normal tissue was very evident with Verhoeff's stain showing purple or black collagen and yellow epidermis in the burned area, and yelow collagen with gray epidermis in normal tissue. Background color was rose or light red.

Hematoxylin-eosin stain showed a deeper purple or reddish color in the epidermis of the burned tissue, but not enough difference in shading to show varying degrees of burn.

The contrast seen in Verhoeff's stain enabled rapid reading of large numbers of slides and permitted the grading of burns with more ease.

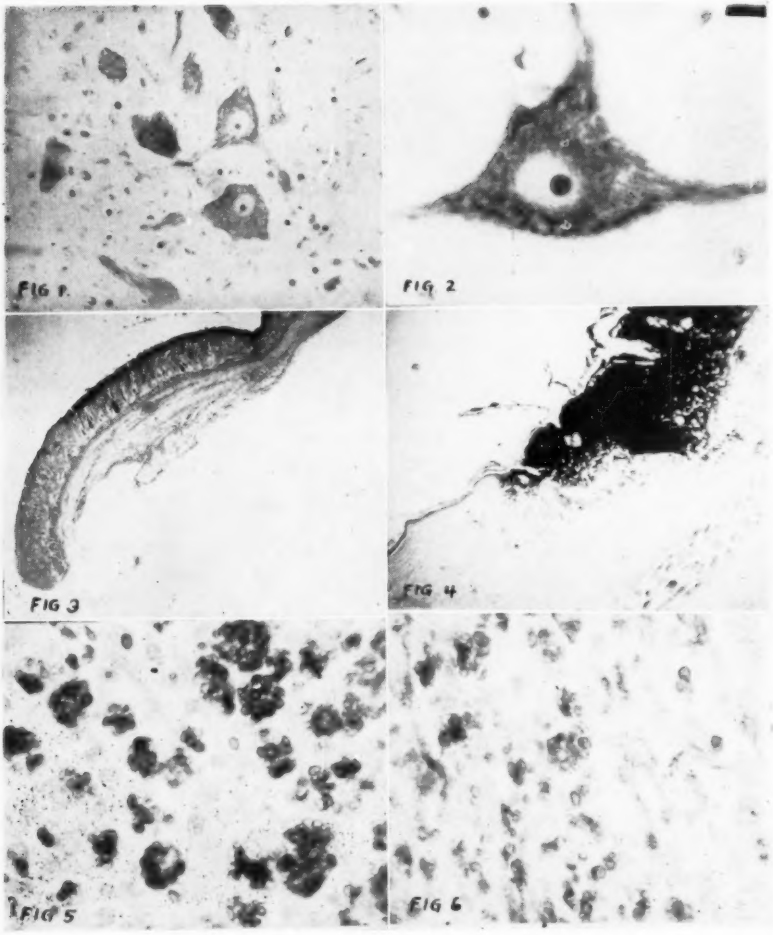

Fig. 1. Brain stem: cat. Nerve cells with Nissel substance in cytoplasm.
Fig. 2. Single nerve cell from medulla oblongata. Lillie's Azure-eosin. Oil, X 915.
Fig. 3. Rat skin, showing burned area on top, normal on bottom. Verhoeff-Van Gieson stain. X 5.4.
Fig. 4. Junction of burned and normal epidermis from rat. Dark area shows severe burn. Verhoeff-Van Gieson stain X 32.
Fig. 5. *Histoplasma capulatum* in lung tissue; dog. Gomori's silver stain. Oil, X 915.
Fig. 6. Same tissue section as Fig. 5 with Gridley fungus stain. Oil, X 915.

III. *Fungus Detection*

Discussion. When laboratory animals are used for research, they are carefully examined when purchased and quarantined for a given period to see that they are disease-free, before being used for experiments. Histology here is applied to a diagnostic problem in keeping the animal colony healthy, rather than a research problem.

If an animal dies and necropsy shows lung disease, it is important to exclude tuberculosis and diagnose fungus infection, if present, as T.B. is contagious, while systemic fungus disease is not easily transmitted. A fungus disease closely resembling tuberculosis in symptoms and gross appearance of tissues is *Histoplasma capulatum*. This organism, which is enzootic in the Ohio River Valley, has been found several times in dogs from the animal colony, and demonstrating it in tissues is about the best means of diagnosis, although it has been reported in cultures.

The clinical signs and gross findings are very similar in both diseases. Tuberculosis is not very common in dogs and cats but may occur in two forms, pulmonary and digestive. In the pulmonary form, the lungs show caseous foci or small miliary nodules, and the bronchial lymph nodes are enlarged. The digestive form shows lesions on the wall of the intestine, liver, spleen, and kidneys. The clinical signs are: emaciation, cough and atypical fever, with diarrhea in the latter stages of the digestive form.[10]

The symptoms of histoplasmosis are parallel. In dogs, persistent diarrhea with intestinal involvment is present. Enlargement of the lymph nodes is characteristic, with occasional gastric ulcerations or gray areas in the liver. In a cat, in which micro-organisms of histoplasma were found, there were enlarged lymph nodes and areas of pseudo-caseation throughout both lungs.[11]

Therefore it becomes necessary to have a good fungus stain which will detect these mycotic organisms in large or small numbers, often within the lesions so similar to tuberculous nodules. The organism appears as a small, oval, yeast-like cell, 3 to 5 microns in diameter,[12] with a narrow capsule. It is often intracellular, being closely packed in collections of large macrophages.

Technique and Results. A silver impregnation technique is recommended by some authors to demonstrate the capsule.[11] One that gives excellent results with a fairly simple and rapid procedure is the Grocott modification of Gomori's silver technique.[13]

The tissue sections are treated with chromic acid, silvered in alkaline methenamine-silver nitrate solution, then placed in gold chloride for toning, fixed in sodium thiosulfate, and counterstained. Aqueous safranin, light green, or metanil yellow may be used for counterstaining. Fungi are sharply outlined in black against the background. Elastic fibers, epithelial mucin, and cartilage also stain black. The silver technique not only aids in detecting fungi but produces a color contrast which is easily photographed.

Another stain specific for fungus organisms is the Gridley fungus stain,[14] which utilizes the chemical reaction of fuchsin stain with aldehyde substances. The tissue sections are oxidized in chromic

acid, treated with Coleman's feulgen reagent, rinsed in a weak acid, washed thoroughly, and stained in aldehyde fuchsin solution. A counterstain of metanil yellow is used, giving a background of yellow, with rose to purple coloring of fungus organisms. Since mucin, cartilage, and elastic fibers are the only other components to take the purple dye, the fungi are easily found and identified.

Summary

A review of special histological stains is given, with their application to research using laboratory animals.

Azure-eosin stain, as used by Lillie, was found very satisfactory for brain tissues, particularly nerve cells.

The Verhoeff-Van Gieson stain, applied to burned tissue, is highly effective for color contrast, rapid reading, and gradation.

The Grocott modification of Gomori's silver technique and the Gridley fungus stain show fungus organisms, usually difficult to detect and stain, clearly and distinctly.

Acknowledgment

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DETECTING BLOOD COAGULATION DEFECTS IN THE CLINICAL LABORATORY*

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Almost every laboratory large or small is now doing the quick one stage prothrombin determination, because of the great demand with the increased use of anticoagulants. The purpose of this paper is to discuss tests which can be valuable in detecting blood coagulation defects and which can be performed by any laboratory which is able to do Quick's prothrombin time determination.

The end step in coagulation is the enzymatic action of thrombin in converting fibrinogen to fibrin. This indicates that an absence or deficient amount of fibrinogen would result in defective coagulation. We know fibrinogen is in the peripheral blood and so then logically thrombin cannot be present without intravascular clotting. Consequently the majority of clotting defects lie somewhere in the formation of thrombin from prothrombin or its derivatives.

There are two available pathways for the conversion of prothrombin to thrombin. One of these pathways involves the action of tissue thromboplastin, calcium, ac-globulin (labile factor) and autoprothrombin I (stable factor) on prothrombin to yield thrombin. In the second pathway prothrombin acts with the antihemophilic factor and autoprothrombin II (PTC or Christmas factor), ac-globulin and calcium. We know the absence, defectiveness or inhibition of any of these factors can result in defective clot formation. Another factor which also must be considered is fibrinolysis. This involves a lysis of the clot after its normal formation.

Procedure

In any examination of this type the results can only be as good as the specimen; therefore accuracy begins by obtaining a good specimen. This involves a clean venipuncture with as little trauma as possible; if plasma is desired blood must be mixed with the anticoagulant immediately after it is drawn. If a clot is visible no matter how small, we have no way of knowing what invisible clotting processes have occurred and the results will be doubtful or erroneous.

The quantitative test for fibrinogen is a biochemical one. The method of Folin-Ciocalteu[6] is used in our laboratory. A low fibrinogen level in the blood will give a slightly prolonged Quick prothrombin time and the end point, clot, will not be solid.

The other clotting components can only be measured by their activity. They are the more common causes of clotting difficulties and they are the ones which are to be discussed in this paper.

It is now an accepted and well known fact that the Quick prothrombin time does not measure prothrombin; however this fact does not alter the value of this test. A prolonged time could be due to a low fibrinogen level or to deficiencies of prothrombin or its accelerators, which are ac-globulin (labile factor), autoprothrombin I (stable factor), or to a circulating anticoagulant. This test does not tell us the specific factor

* Read before the Annual Convention, Chicago, Illinois, June 1957.

causing the difficulty; however if we could add a specific factor to the test plasma and correct the defect we could then assume that factor to be the defective one. Aged, adsorbed rabbit plasma will correct an ac-globulin (labile factor) deficiency. Fresh, normal plasma will correct any of these deficiencies but it will not correct a circulating anticoagulant. Circulating anticoagulants are found to be associated with Lupus Erythematosus.[4]

The most common use of this prothrombin time determination is in following anticoagulant therapy. Most anticoagulant drugs reduce the levels of prothrombin, autoprothrombin I and autoprothrombin II[8]; however an exception is "marcumar" in that it does not affect autoprothrombin II.[7] Heparin interferes with the conversion of prothrombin to thrombin; therefore the prothrombin test following heparin therapy is erratic. The results of the test depend on the time the blood was drawn in respect to the administration of heparin. If the specimen is drawn soon after the administration of heparin, the prothrombin time may never give an endpoint and if a specimen is again drawn in four to eight hours after the heparin has been metabolized the prothrombin time may be 100 per cent of normal.

The difficulty in hemophilia occurs in the action of either the anti-hemophilic factor or autoprothrombin II in the formation of plasma thromboplastin.[3] In the prothrombin time test thromboplastin is added and so this step is obscured and as a consequence the prothrombin time is normal in classic hemophilia and cases of deficient autoprothrombin (PTC or Christmas factor).

The plasma clotting time or recalcification time is a helpful test to use in picking up defective clotting especially of the hemophilic type. In this test the calcium in oxalated plasma is replaced and the clotting time determined. This test involves the whole blood clotting process so it could take the place of a Lee White or other whole blood clotting tests. It has some advantages over them because it is done on oxalated blood; it can be done in duplicate without inconveniencing the patient; it is more reproducible because of the sharp end point; and it can be done at the convenience of the technician.[9]

A prolonged plasma clotting time is found in cases of classical hemophilia, autoprothrombin II (PTC or Christmas factor) autoprothrombin I (Stable factor), ac-globulin, prothrombin and fibrinogen deficiencies, and cases of circulating anti-coagulants.

The method of adding a specific factor mentioned before is adaptable to the plasma clotting test and especially helpful in differentiating classical hemophilia and autoprothrombin II deficiencies. If fresh, normal, $BaCO_3$ adsorbed plasma will shorten a prolonged plasma clotting time, hemophilia is indicated and autoprothrombin II (PTC or Christmas factor) deficiency can be ruled out. Neither aged human plasma nor serum will correct hemophilia but both will correct autoprothrombin II deficiencies.[1, 2, 13] If the addition of fresh normal plasma does not shorten a long plasma clotting time an inhibitor is indicated. Adsorbed rabbit plasma will correct a deficiency of ac-globulin (labile factor).

The prothrombin consumption test is an indication of the rate prothrombin is utilized during clotting. Abnormal consumptions are found

in cases of hemophilia, thrombocytopenia, functional platelet deficiencies, and inhibitory substances in the blood. The method of correcting that has been used to identify deficient factors in the prothrombin time and the plasma clotting can not be adapted to this test. For this reason in our laboratory we find it helpful to do the prothrombin time and plasma clotting time whenever a consumption is done and then we can carry out the correcting procedures if indicated.

Reagents and Methods

Adsorbed rabbit plasma, which will serve as a source of ac-globulin.

Kill rabbit by venous injection of air. Rapidly open and remove blood from the heart and immediately mix nine parts of blood with one part 0.1 molar sodium oxalate. Centrifuge and remove plasma. Adsorption is accomplished by using moist $BaCO_3$. Weight $BaCO_3$ 50 mg. per ml. plasma, add a few ml. saline, centrifuge and discard the supernatant. Add plasma to the moist $BaCO_3$ and shake in a 37° C waterbath for ten minutes. Remove the $BaCO_3$ by centrifugation, put the plasma in small tubes and store in the deep freeze.

Adsorbed fresh, normal plasma which will contain ac-globulin and the antihemophilic factor

Adsorb with barium carbonate in the same way as the rabbit plasma. A Quick prothrombin time on the adsorbed plasma of over 60 seconds indicates that the adsorption is sufficient. This preparation must be made from fresh plasma whenever it is needed because both the ac-globulin and antihemophilic factor disappear on storage.

Aged human plasma which will contain autoprothrombin II (PTC or Christmas factor)

Draw plasma off the cells of human blood and expose the plasma to the air at room temperature for several days. As soon as the prothrombin time by Quick's method is over 60 seconds (usually 4 to 6 days) the plasma is aged. It should then be put in small tubes and stored in the deep freeze.

Human serum which will contain autoprothrombin II (PTC)

Blood is allowed to clot at 37° C for two hours, then centrifuged and the serum drawn off and then allowed to remain at room temperature for 24 hours. Then it is tubed and stored in the deep freeze.

Calcium chloride 0.025 molar

Thromboplastin, Difco rabbit brain made according to their specification. Simplastin or acuplastin or any prepared thromboplastin-calcium chloride reagent may be substituted.

Fibrinogen, Warner-Chilcott, make according to their specifications.

Sodium oxalate 0.1 molar. Used as an anticoagulant in proportions of one part to nine parts whole blood.

Methods

Quick one stage prothrombin time[10, 11]

A small tube containing 0.1 ml. thromboplastin and 0.1 ml. calcium chloride is placed in a 37° C waterbath. Blow into this mixture 0.1 ml. plasma, and start a stopwatch simultaneously and determine the clotting time. All tests are done in duplicate or until results agree within one second. By this method normal plasma gives a prothrombin time of 12 to 15 seconds.

Plasma clotting time. Owen's[9] modification of Biggs and MacFarlane's test.[3] Blow 0.2 ml. plasma into 0.2 ml. calcium chloride and simultaneously a stopwatch is started and the clotting time determined. Normal is 90 to 150 seconds.

Prothrombin Consumption

Draw blood and allow to clot at room temperature. As soon as the blood has clotted by the Lee White method place it in a 37° C waterbath for exactly 50 minutes. Centrifuge 3 to 4 minutes. Remove serum and place in waterbath for five minutes. During this last ten minute period make up a mixture of two parts thromboplastin and one part fibrinogen and put 0.2 ml. of this mixture in small tubes and place in the waterbath. Now when the serum has been in the waterbath five minutes it will be exactly 60 minutes after the blood clotted. Immediately add 0.1 ml. serum to the thromboplastin and fibrinogen mixture in the waterbath and simultaneously start a stopwatch and determine the clotting time. Normal is a clotting time of over 30 seconds; abnormal is under 20 seconds; and the time of 20 to 30 seconds falls in the doubtful category.

Fibrinolysis

A drop of thrombin is added to 0.2 ml. plasma in a small tube to form a clot. A control using normal plasma is always done along with the speciman being tested. The clots are placed in a 37° C waterbath. At intervals of 15 or 30 minutes both tubes are examined and compared for lytic activity.

Correct

In attempting to correct the plasma clotting time or the prothrombin time one part of the correcting plasma or serum is added to nine parts of the patient's plasma and the test then done on the combination in the same manner as before. The results are then compared. If the deficient factor in the patient's plasma is in the added substrate the prolonged test will be corrected to normal but it will have to be markedly improved in order to call the added factor the deficient one.

If a circulating anticoagulant is present in the patient's plasma, one part of that plasma added to nine parts of normal plasma will prolong the plasma clotting or prothrombin time of the normal.

Review of Cases

Blood specimen #1

Lee White Clotting time: 16 and 17 minutes
Prothrombin time: 15 seconds, 87%
Prothrombin consumption: 26 seconds
Plasma clotting time: 195 seconds
One part normal plasma to 9 parts specimen gave a plasma clotting time: 160 seconds
One part human serum to 9 parts specimen gave a plasma clotting time: 140 seconds
Report: Autoprothrombin II (PTC) deficiency.

Blood specimen #2

Lee White clotting time: 26, 49, and 67 minutes
Prothrombin time: 21 seconds, 48%
Prothrombin consumption: 18 seconds
Plasma clotting time: over 20 minutes

One part normal plasma to 9 parts specimen. Plasma clotting time: over 20 minutes
One part human serum to 9 parts specimen. Plasma clotting time: over 20 minutes
Nine parts normal plasma to one part specimen. Plasma clotting time: prolonged to 315 seconds
Report: Presence of circulating anticoagulant.

Blood specimen #3

Lee White clotting time: 10½ and 12 minutes (poor clot)
Prothrombin time: 17 seconds, 82%
Prothrombin consumption: 45 seconds
Plasma clotting time: 140 seconds
Fibrinogen 79.5 mg.%
Plasma tested for fibrinolysis
0.2 ml. plasma plus one drop thrombin. Clot formed slowly and at the end of 30 minutes in 37° C waterbath the clot was nearly all lysed.
0.2 ml. normal plasma was treated in the same manner to serve as a control; however at the end of 30 minutes no lysis was apparent.
0.1 ml. plasma was mixed with 0.1 normal plasma and treated in the same manner; at the end of 30 minutes lysis had occurred but not as marked as that of the specimen #3 plasma alone.
Report: fibrinolysis.

Blood Specimen #4

Lee White clotting time: 23 and 34 minutes
Prothrombin time: 16 seconds 90%
Prothrombin consumption: 7.8 seconds
Plasma clotting time: 405 seconds
One part human serum and 9 parts specimen. Plasma clotting time: 180 seconds
One part human serum and 9 parts specimen Plasma clotting time: 375 seconds
One part fresh $BaCO_3$ adsorbed normal plasma and 9 parts specimen Plasma clotting time: 170 seconds
Report: Deficiency antihemophilic factor.

Summary

A brief review of the factors involved in blood clotting is presented. The value and limitations of coagulation tests is discussed along with a brief outline of methods. The process of isolating a defective factor by adding plasma or serum, which contain known factors, to the patients plasma and trying to correct abnormal prothrombin times or plasma clotting times is discussed.

Four blood specimen from our laboratory and a report of our findings is given.

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ANNOUNCEMENTS

The NOMINATIONS COMMITTEE announces the expiration of the terms of office in June 1958, of the following incumbents:

President-elect: Elizabeth O'Connor
Recording Secretary: Nellie Mae Bering
Board of Directors:
Ruth Heinemann
Sister Mary Simeonette Savage
Board of Schools:
Mary Frances James

RADIOISOTOPE TECHNIQUES FOR CARDIAC AND BLOOD STUDIES DESCRIBED IN REPORT

A radioisotope technique for determination of cardiac output which is said to offer "virtually unlimited potential" in the diagnosis and control of heart disease is described in a report of Air Force-sponsored aeromedical research just released to the public through the Office of Technical Services, U. S. Department of Commerce.

The non-traumatic method utilizes injection doses of radioionated human serum albumin (RISA). Simple and highly accurate, the technique can be used to demonstrate precisely the variations of cardiac output under stress and response to medications, according to the report. It is reproducible to about 6.0 percent in individual patients.

Also described is a method for measuring the volume of whole blood, plasma, and red cells which eliminates the complex plasma separations and washing and drying of erythrocytes characteristic of earlier approaches. The venous hematocrit determined by RISA is utilized to compute the specific activity of chromium-51 tagged red cells. Through use of a concentrated ACD (C-ACD), erythrocyte tagging, injections and sampling can be completed in about 35 minutes.

The report, PB 121984 *Blood Volume and Cardiac Output Determinations Using Radioisotopes*, R. E. Zipf, J. M. Webber, and G. R. Grove, Miami Valley Hospital, and T. F. McGuire, Wright Air Development Center, Nov. 1956, may be ordered from OTS, U. S. Department of Commerce, Washington 25. It contains 35 pages, price $1.

DETERMINATION OF SERUM CHOLESTEROL

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The ferric chloride reaction[1, 4, 5, 11] for the rapid, simple analysis of the cholesterol content of serum has seen extensive and reliable use in our laboratories during the past several years. At the present time there has been much greater emphasis placed on the need and ability to employ less material in clinical work. We have therefore adapted the procedure with some modifications to the determination of the total and free cholesterol of smaller samples than previously described. The technic is similar and maintains the simplicity involved with larger sample analysis.

Several phases of the previous work were investigated further in the present study aside from the use of less serum and these involved widening the range of analysis without the necessity of repeating the entire procedure, an improved reagent technic for an increase in reagent stability, room temperature extraction of the cholesterol, mechanism of color formation, and temperature control during color formation.

Reagents and Equipment

Ferric chloride stock reagent. Weigh out 700 mg. of C.P. $FeCl_3 \cdot 6\ H_2O$ on an analytical balance and dissolve it in glacial acetic acid in a 100 ml. volumetric flask. Dilute to the mark of the flask and mix the solution well by inversion.

Ferric chloride working reagent. Dilute the stock reagent 1:10 with glacial acetic acid.

Cholesterol stock standard. Dissolve 100 mg. of pure dry C.P. cholesterol in glacial acetic acid in a 100 ml. volumetric flask and dilute the solution to the mark with glacial acetic acid.

Digitonin solution. Dissolve 1 g. of digitonin in 50 ml. of ethanol and dilute the solution to 100 ml. with distilled water.

Extraction solution, alcohol-acetone. Prepare a 1:1 mixture.

Acetone, C.P.

Concentrated sulfuric acid, A.R.

Coleman Jr. Spectrophotometer, Model 6A and 1 cm. cuvets.

Procedure

Preparation of Calibration Curves: Prepare a fresh cholesterol working standard by diluting 1 ml. of the ferric chloride stock solution and 1 ml. of the cholesterol stock standard to 10 ml. in a volumetric flask with glacial acetic acid. Pipet 0.0, 1.0, 2.0, and 3.0 ml. of this standard solution into test tubes and dilute each to 3.0 ml. with the ferric chloride working reagent. Carefully layer in 2.0 ml. of concentrated A.R. sulfuric acid and mix the solutions thoroughly. When they have cooled to room temperature, approximately 10-15 minutes, measure their absorbances at 560 mu against the blank.

Determination of Serum Cholesterol: Pipet 0.2 ml. of serum into a 10 ml. volumetric flask containing about 5.0 ml. of acetone-alcohol extraction solution, dilute to the mark with vigorous shaking to effect extraction and filter the mixture through Whatman #41-H filter paper, keeping the funnel covered with a watch glass. Pipet 5.0 ml. of the

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filtrate into a conical centrifuge tube for the free cholesterol determination and 2.5 ml. of filtrate into a test-tube for the total cholesterol determination.

Evaporate the contents of the test tube to dryness and the contents of the conical centrifuge tube to approximately 0.5-1.0 ml. Add 3.0 ml. of ferric chloride working reagent to the test tube to dissolve the residue, layer in 2.0 ml. of concentrated sulfuric acid and mix the solution thoroughly. After the appropriate waiting period measure the absorbance against a blank at 560 mu. Add 0.5 ml. of the digitonin solution to the contents of the centrifuge tube, wait 15 minutes, centrifuge the tube at 3500-4000 RPM for 10 minutes and carefully decant the supernatant fluid. Blow in four ml. of C.P. acetone to disperse the precipitate, tap the tube until the precipitate is homogenous, and then recentrifuge for 10 minutes before carefully decanting the wash supernatant fluid. Allow the inverted tube to drain on absorbent paper before adding ferric chloride reagent and sulfuric acid for measurement in the manner previously described. Experimentation with standards showed that the final color formed stoichiometrically up to 1000 mg. per 100 ml. of total cholesterol and that the range of analysis could be extended to include most of the extreme pathological cases by an appropriate dilution of the sample with the blank.

Discussion and Results

Over a period of time, especially during warm weather, it was found that a precipitate formed in the sulfuric acid-ferric chloride reagent which necessitated the preparation of new reagent. This was probably due to a loss of hydrochloric acid with the subsequent formation of the less soluble ferric sulfate. However, by dissolving the ferric chloride in glacial acetic acid, the hydrochloric acid did not appear to be lost, no precipitate formed and the iron content remained constant for the greater than a month period during which the reagent was analyzed.[12] The sulfuric acid color reagent originally contained 2.0 mg. of the $FeCl_3 \cdot 6\,H_2O$ in the 2 ml. volume used, whereas the glacial acetic acid reagent contains 2.1 mg. in the 3 ml. volume used. Absorbance values obtained for the two reagents compare favorably when they are reacted with the same amount of cholesterol, so that small differences in iron concentration are comparable for carrying out the reaction. It is important however to maintain the concentration of iron near the vicinity of a plateau with respect to color formation for accuracy since significant lowering of the iron content decreases the amount of color formed.[11]

Several concentrations of cholesterol were reacted with ferric chloride and the ferrous iron contents of the resulting solutions were determined. It was found that the ferrous iron values increased as the cholesterol concentration increased indicating that the color formed might be due in part to an oxidation, possibly in conjunction with dehydration of the cholesterol molecules since the sulfuric acid concentration of the final nonaqueous solution was forty percent. Dehydration and oxidation of organic molecules by sulfuric acid or sulfonic acids have been investigated and reported by Feigl.[2] Nath, et al,[6,7] have shown that cholesterol can be dehydrated and sulfonated by sulfuric acid in acetic acid to form a sulfonic acid derivative of cholesterilene. Solvation is a recognized phenomenon whereby colored compounds form as addition products of

sulfuric acid depending on the coordination ability of the atoms or groups of the compound with the sulfuric acid. The color can be removed on dilution with water.[3] It is possible that the oxidized form of cholesterol, may be dehydrated by sulfuric acid (or the order of the two reaction steps may be reversed) and the compound formed then solvates with sulfuric acid. There is a possibility therefore of sulfonation, solvation, dehydration and condensation, or oxidation being involved in the color formation.

The temperature of the reaction mixture during the formation of color has not been rigidly controlled although small changes in temperature did not appear to affect the final color with the concentrations of cholesterol employed.[11] On mixing sulfuric and acetic acids in the ratio of 2:3 respectively, a temperature of approximately 67° C was found. Varying concentrations of cholesterol in the correct amount of working ferric chloride solution and sulfuric acid mixture were iced to prevent color formation and then warmed at 50° C, 60° C, 70° C and 100° C respectively in a water bath for five minutes. The measured absorbances at 60° C and 70° C were similar to those obtained when the solutions were mixed without prior icing.[11] However the 50° C and 100° C samples measured at smaller absorbance values, indicating that the color for the lower temperature had not completely formed whereas the color at the higher temperature had been partially destroyed. Although others had found that a constant temperature gave more reproducible results[4] the sample size involved was much smaller which must be an important factor. Our results indicate that the use of a constant temperature device for cholesterol in the ranges determined in the described procedure does not appear to be necessary since the reaction takes place in the 60°-70° C range when mixing without temperature control is employed.

In order to insure accuracy of recovery with mixtures, a number of standards were made up in alcohol-acetone which contained varying amounts of cholesterol as well as cholesterol acetate. Aliquots were examined for both constituents and the results obtained are shown in Table I. A good correlation is shown between the values known to be present with the values determined by the quantitative procedure described.

TABLE I

Recoveries Made on Varying Mixtures of Cholesterol and Cholesterol Acetate

Milligrams per 100 ml.

Total Cholesterol Present	Cholesterol Acetate Present	Total Cholesterol Found	Cholesterol Acetate Found
250	50	250	53
250	50	254	53
250	100	245	105
250	100	257	97
250	150	245	153
250	150	256	148
400	100	398	96
400	100	403	103
400	200	391	196
400	200	400	197
500	100	500	95
500	100	500	104
500	200	491	197
500	200	502	193
700	200	677	190
700	200	692	206
700	300	692	307
700	300	691	298

Some cholesterol procedures use room temperature extraction for the desired constituent, but the Schoenheimer-Sperry procedure[8] and its modifications[9,10] using alcohol-acetone as the solvent apparently inferred that it was necessary to heat the solution to effect complete extraction. It was decided to compare duplicate analysis samples where one sample was heated while the other sample was not heated. The results obtained, shown in Table II, indicate that the similarity in the comparison warrants the elimination of the heating step.

TABLE II

Comparison of Serum Cholesterol Values on Heated and Unheated Samples

Heated, mg. per 100 ml.		Unheated, mg. per 100 ml.	
Total	Free	Total	Free
312	100	314	97
222	106	220	100
236	91	244	86
302	114	288	117
572	214	572	210
256	111	246	106
274	86	274	91
256	106	244	111
212	69	222	69
188	52	188	54
214	67	216	71
180	54	186	56
212	63	200	66
226	114	228	114
166	83	166	77
228	60	226	58

Table III shows the comparison data obtained on a number of serum samples for the procedure described here and the previous technic which it modifies.[10] The difference in the results of the two analyses on each sample indicates that the cholesterol content of the smaller sample is adequately determined. For total cholesterol determinations alone as little as 50 microliters of serum can be successfully handled.

TABLE III

Analytical Comparison of Old[7] and New Procedures for Total and Free Cholesterol in Serum

Total Cholesterol Found, mg./100 ml.			Free Cholesterol Found, mg./100 ml.		
Old	New	Diff.	Old	New	Diff.
163	161	2	51	57	6
177	180	3	51	52	1
186	190	4	66	64	2
168	170	2	77	80	3
177	176	1	60	64	4
212	206	6	57	63	6
343	348	5	97	94	3
245	252	7	61	63	2
140	148	8	40	41	1
414	428	14	120	122	2
212	212	0	58	60	2
189	189	0	54	50	4
177	176	1	60	64	4
190	186	4	64	66	2
170	168	2	52	51	1

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ABSTRACTS

ISOLATION OF THE INFECTIOUS BOVINE RHINO-TRACHEITIS VIRUS

Stewart H. Madin, Charles J. York and Delbert G. McKercher (University of California and Pitman Moore Company, Indianapolis) Science, 124, 721 (1956).

A disease characterized by sudden onset, pyrexia, abrupt cessation of milk flow, salivation, dyspnea, and severe inflammation of the upper respiratory passages and trachea, occurred in dairy cattle of California in 1953. Again in 1954 and later. Reports from Colorado were similar. Virus isolates, of either source were identical antigenically. The etiological agent will pass through a fine sintered glass filter and will survive storage at —70° C for at least 7 months and at 37° C for at least 96 hours. The condition of the disease caused by this virus was called "infectious bovine rhino-tracheitis" (IBR). Various studies indicated that other micro-organisms could not be incriminated. A careful search of infected tissue culture fluids failed to reveal pleuro-pneumonialike organisms (PPLO) when cultured on standard PPLO media by the method of Adler et al.

SOME OF THE VARIABLES INVOLVED IN THE FRACTIONATION OF SERUM PROTEINS BY PAPER ELECTROPHORESIS

Richard J. Henry, et al., (Bio-Science Laboratories, Los Angeles), Clin. Chem. 3, 49-64 (1957).

The investigation reported in this paper is a study of what appear to be major variables involved in a single standardized technic. The apparatus used was the inverted V form marketed by the Spinco Division of Beckman Instruments, Inc.

The paper is divided into parts dealing with variables in electrophoretic separation, variables in staining, quantitation of stained electrophoretic strips, stability of sera, standardized procedure, and normal values. Under variables in electrophoretic separation the authors discuss paper, ionic strength of buffer, size and protein concentration, and time and voltage. Under variables in staining; heat-fixation of the proteins, different lots of dye as to time, temperature, age, and washing and drying of strips. Each of these discussions is followed by a summary and in some instances two technics may be compared.

SERUM PROTEIN TURNOVER IN MULTIPLE MYELOMA

Solomon A. Berson and Rosalyn S. Yalow (V.A. Hospital, Bronx), Jour. Lab. and Clin. Med. 49, 386-394 (1957).

The study reported in this paper was designed to determine the rate of turnover of myeloma protein labeled with I^{131} and to evaluate in another way the possible derivation of Bence-Jones protein from serum myeloma protein. The serum proteins of a patient with a high serum concentration of myeloma globulin but no Bence-Jones proteinuria were labeled with I^{131} and administered intravenously to a patient who showed no abnormal serum proteins, but yet excreted approximately 15 to 20 gms. of Bence-Jones daily.

The serum myeloma protein pool in the donor subject was about two and one-half times that of the recipient or normal subjects. The daily turnover of serum myeloma protein (36 gms. per day) is approximately two and one-half times that of serum albumin in normal subjects. The urine of the donor subject never exhibited any significant quantity of protein-bound radioactivity. Only about 5% of the radioactivity in the urine of the recipient subject was precipitable, indicating that the major portion of the labeled serum had been subjected to metabolic degradation prior to excretion of the I^{131}.

This investigation does not support the concept of a precursor-product relationship between serum myeloma and Bence-Jones protein, respectively, but the possibility that Bence-Jones protein is a procursor or abortive product of serum myeloma globulin synthesis is not excluded.

NEW APPLICATIONS OF THE LUXOL FAST BLUE MYELIN STAIN

George Margolis and John P. Pickett, Lab. Invest. 5, 459-473 (1956).

Luxol fast blue myelin stain (du Pont) is a new myelin stain based on the use of an alcohol-soluble amine salt of sulfonated copper phthalo-cyanine. This dye is characterized by the intensity of the blue or bluish-green staining, its specificity for myelin, and its remarkable fastness to light, heat, acids, and alkalis. It is applicable to formalin-fixed tissues without the usual mordants required for the traditional myelin methods and of being adaptable to paraffin, celloidin, and frozen sections. Further the dye can be used in combination with numerous other stains to bring out the cellular elements.

Four staining procedures are outlined in this article using the Luxol fast blue myelin stain of Kluner and Borrera. A combined myeloangio-cytoarchitectonic method, a myelin-neuroglia stain, a myelin-fast technic, and a myelin-axis cylinder method are described. The simplicity and economy of the described technics provide an opportunity for the incorporation of these definite neurohistologic technics into the methodology and teaching in any modern pathology laboratory.

ULTRAMICRO METHOD FOR SERUM BILIRUBIN BY DIAZO BLUE REACTION

Ramon E. Stoner, et al. (Mount Sinai Hospital and Chicago Medical School, Chicago), Clin. Chem. 3, 22-35 (1957).

The method described in this paper to determine total serum bilirubin requires from 0.02 to 0.05 ml. of serum using the Coleman Junior Spectrophotometer, Model 6A with 12 x 75 mm. round cuvettes. The method is a modification of the (acid) diazo blue reaction used by Thannhouser and Anderson with precipitation of the serum proteins. A minimum of 1.5 ml. of the supernatant is read at a wavelength of 580 mu. Included in the text are absorption and calibration curves of the blue azobilirubin using the Coleman Junior Spectrophotometer and the Evelyn with a microcell attachment. Complete recovery studies show that within the limits of experimental error, 1 microgram of bilirubin would be recovered for each microgram of the calculated value for a specific serum in this series of analysis.

The method is reproducible and sensitive, being carried out in final serum dilutions of 1:100 to 1:40. This method compares favorably with the Malloy and Evelyn method which was done in parallel.

MEDICAL TECHNOLOGY: PROFESSION VS. OCCUPATION*

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Religion, medicine and law are called the "learned professions." The occupations of teaching and military service are also regarded as professions. What distinguishes these fields from say, business or farming? A man may devote just as much time and attention to his business as another does to his profession. Both earn their living by engaging in their own particular work. Yet a few occupations are singled out by society as requiring a special calling or dedication to duty above and beyond the average in the service of their fellow man. These are the professions.

The medical technologist engages in work of vital service to humanity. Upon his skill and integrity may depend the very life of another. Upon his technical aid to the doctor may depend the rapid and successful diagnosis of the sick, and their prompt and proper treatment. His calm attitude and cheerful manner reassure the patient at a time of personal crisis. To the patient, the technologist represents medical science in action for his own special benefit. The nature of the technologists' duties and responsibilties require maturity of mind, warmth of heart, and skill of hand. He is dealing not in dollars, or produce, or other material things, but in the most precious of commodities, human life.

We reason correctly that our occupation demands more mentally, spiritually, and even physically, than the usual job, and so we justly and proudly consider ourselves members of a fine profession—the profession of medical technology.

It is a rude shock, then, to find that among many usually well-informed people, we are not so regarded. The Armed Forces, for example, do not give us any special consideration as a group; yet nurses have long since been recognized as professional workers, and are given commissions on the basis of their training. In most states, chiropractors, barbers, and food handlers are looked upon as specialized groups requiring licensure for the public good. Medical technologists in the majority of states are not required by law to meet any sort of minimum standards of education or training. Most states do not recognize medical technologists as a professional group at all.

Why are we not generally regarded as professional workers? Since World War II with the increasing scarcity of well-trained laboratory personnel, and the spiraling demand for more and more laboratory tests all over the country, our group of registered technologists has been wellnigh overwhelmed by the influx of informally trained laboratory workers who are called laboratory technicians too. An article in "Medical Economics" in 1955 reported that there are over 50,000 people working in clinical laboratories throughout the country. Of these, some 20,000 are actively working registered technologists with at least some similarity of training for their jobs. The others represent all levels of training; ranging from grade school to college graduates. Many of them may be as competent as many registered technologists, but at present there is no

* Read by title—Annual Convention—Chicago, Illinois, June 1957. Published "Kansas Scope," June 1957.

universally recognized way to measure their relative competency. If it were certain that inexperienced laboratory workers attempted to serve only as aides to qualified personnel and truly worked under close supervision, the situation would be less disturbing. But in the present crisis, it is evident that many attempt to run intricate chemical analyses, set up cross-matches, or do other highly complicated and critical work beyond their capabilities, and largely on their own. The existence of a group of workers of such diverse educational backgrounds and varying degrees of competence all doing the same type of work, and all considered laboratory technicians, is the main stumbling block to our professional recognition.

In the past, the American Society of Clinical Pathologists attempted to set up uniform standards for laboratory personnel, requiring a certain amount of college work, at least a year of interneship in an approved school, and the ability to pass a national examination given by the Registry of Medical Technologists. Since the scope of the technician's duties has constantly widened, and the demands made upon him have multiplied with each new scientific advance, this minimum course of study seems short enough. And yet the sad fact remains that many medical employers apparently do not know of the Registry, or, worse still, do not feel that such registration is important. We demand that plumbers and electricians attain a certain level of competence before we turn them loose on our water pipes and wires. Yet it seems we are willing to trust our lives to amateurs in the laboratory.

Since registration of the medical technologist is on a voluntary basis, and since medical employers are not bound by the recommendations of the Registry, thinking technologists have been considering more compulsory forms of regulation, either on the state or national level. Several states have already taken steps to control the quality of their laboratory workers. Our American Society of Medical Technologists is currently investigating the pros and cons of state licensure for other areas, before committing its membership to a definite course of action. Clearly, the present confusion regarding who is and who is not a medical technologist must be resolved if we are ever to function coherently as a true profession.

Why are we, as registered technologists, so concerned with this problem of professional status and standards? We are concerned because we are proud of our group, and justly so. We want others to regard us as we regard ourselves. But it is not simply a matter of pride. It is a matter of serious responsibility. It is our duty to emphasize again and again to members of the medical profession that the medical technologist is a vital member of the medical staff, and must be just as well trained as the registered nurse. It is probably true that many mothers would make excellent nurses. It is probably true also that any girl who can bake a lemon pie can be a good technician. Yet both actually require the benefit of training under qualified teachers to prepare themselves adequately for either type of work.

We are concerned with the standards of medical technology also because every technician suffers from the poor work of another. And obviously, poorly trained personnel are more likely to make mistakes. Such

workers, unwittingly or otherwise, by the variability of their technical competence, tend to discredit all laboratory workers and all laboratory procedures. The reporting of inaccurate results over a period of time by untrained or improperly supervised personnel has done incalcuable harm to medical technology in general. It causes many doctors to view unexpected laboratory findings with understandable caution, and may cut off from both doctor and patient the benefits of modern scientific information which should be theirs. While it is true that laboratory tests reflect only a part of the picture of the patient's health, nevertheless, they should be consistently reliable, and of at least supplementary value.

We are concerned with the standards of medical technology also because in many areas the registered technologist, with an investment in years of training and experience, is accorded no more respect or recompense than the casual laboratory worker. The registered technologist regards his work as a real career—not as a stop gap activity between high school graduation and the triumphant walk to the altar. He devotes at least part of his time away from work in reading professional publications in order to keep up with new developments in the field. Even if such technical literature is available to the informally trained worker, he is not likely to be interested in articles unrelated to his job. When such articles directly concern his work, he is usually too unfamiliar with terminology, background information, and theory to benefit fully from such material. The registered technologist usually attends workshops or post-graduate courses at least once a year. The ordinary laboratory worker has little interest in such educational activities, may not be aware of their existence, and is ineligible for many of them. The registered technologist at A.S.M.T. meetings has the opportunity to talk to professional associates working in other laboratories. He may discuss common problems with his fellow technologists and find out how they were solved in other places. The casual laboratory worker rarely has an opportunity to discuss his work with anyone outside his own laboratory. Finally, in many instances, one well-trained technologist would serve where two amateurs struggle, since less time is wasted in any activity that is handled with familiarity, confidence, and skill. And yet in some areas, apparently, registered technologists are considered to be unnecessary luxuries.

The current chaos in the clinical laboratory has affected every registered technologist in some way, but perhaps its most serious effect has been, and will increasingly be, on recruitment. If young people find that they can obtain jobs in laboratories without registration, and at perhaps identical pay, why should they go to the expense and effort of years of proper preparation? Unless such training is valued and universally required by the medical employer, it will vanish. I do not anticipate this tragic consequence of present thoughtlessness. Yet our future progress depends upon the continuing demand for formally trained medical technologists, and their increasing recruitment.

Certainly the most serious single problem facing us is the present shortage of registered technologists. Many of the difficulties we are encountering now are a direct result of the simple fact that there are not enough of us. Many doctors search in vain for a registered technologist.

and since the work must be done, make a reluctant compromise, and hire individuals with some knowledge of laboratory work to fill the jobs. Frequently, in a large hospital, one or two registered technologists are employed where four or five are needed. In order to handle the volume of work in such places, laboratory aides are used. Such measures are born of necessity, but should not cause us to relax our professional standards. In an effort to control somewhat the level of training of laboratory aides, the Registry of Medical Technologists has created a special category for such workers, and has outlined a program for their training. It must be remembered, however, that compliance with suggestions from the Registry is entirely voluntary, and does not affect hundreds of workers who may never have heard of it, nor does it necessarily influence their employers. The fact remains that there is a critical shortage of well trained laboratory workers, and that there is no substitute for such personnel if costs in time lost, money wasted, and suffering needlessly prolonged are honestly measured.

What can the registered technologist do to improve the standards and status of the profession? First of all, he can try to improve himself and the quality of his own work through continued practice and study. One shining example of technical ability and personal integrity is worth more than any argument, however well-presented. He should attend all the workshops, post-graduate courses, and conventions of his A.S.M.T. Society available to him. If the registered technician is plainly superior on the job, she will create respect for her professional training, and demonstrate to the physician the desirability of hiring registered technicians whenever possible.

Secondly, he should call to the attention of his own medical employer the crying need for effective regulation of workers in the clinical laboratory. He may be able to get others in his medical society to consider the problem. If the medical men are thoroughly convinced that what goes on in the laboratory is of critical importance to their successful practice and to the welfare of their patients, they will take appropriate action. It is up to us to convince them.

In addition, the registered technologist should work personally, and through the state A.S.M.T. Society to recruit new members, encouraging them to prepare themselves properly for this career in medicine. He should work for the increase of scholarships to the aspiring technician by telling groups interested in such educational grants about the desperate need for registered technicians. He should work also through his A.S.M.T. Society for the recognition of medical technology as a profession by the Armed Forces and his own state government. State licensure, with definite standards of competency demanded of all laboratory personnel, should be carefully considered by him. It could furnish the necessary control in his particular area. He, above all, should stand firm against those who would lower the educational requirements for registered technologists, as this would most surely undermine the status which we have achieved up to now.

The profession of medical technology is still in its youth. With perserverence and unswerving loyalty to our high ideals, it should shortly be given the acknowledgment it so richly deserves. Yet it will take

patience, hard work, and the education of all members of the medical profession and the public on the part of each one of us to reach our goal. Perhaps one day in the future, the man on the street may be able to distinguish us from waitresses or beauty operators. And perhaps the medical men themselves will rank us more equally with other members of the medical team. It is up to us to earn for our profession this recognition.

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AMONG THE NEW BOOKS

SURGEONS ALL, by Harvey Gresham, M. D., Philosophical Library, Inc., New York, N. Y., 1957. 4159 pp. 29 illustrations. $10.00.

A thoroughly fascinating history of surgery from prehistoric times when the surgeon's knife was a "flint newly flaked," through developments in early Greece and Rome. The renaissance of surgery in the Middle Ages and that in Elizabethan times, are mentioned—all in an easily read style.

As early as 1308 there was a return to dissection and lectures in anatomy. Barber-surgeons had their apprentices in the 1500's.

The work of William Harvey* is described in the chapter entitled "A One-Man Revolution." Shortly after his death the surgery was separated from barbering. The contributions of Lister and the development of anaesthesia lead to the surgery of "Today and Tomorrow," with the "Postscript" carrying on to 1956. The book ends on the note of "And it is a story full of hope that remains always unfinished . . ." *Surgeons All* will be enjoyed by the layman and professional alike.

WILLIAM HARVEY,* HIS LIFE AND TIMES: his Discoveries; his methods, by Louis Chanvois, Lauréat de l'Institut et de l'Académie de Medécine. Translation. Philosophical Library, Inc., New York, N. Y. 1957. 270 pp. 18 Plates. 14 line illustrations. $7.50.

A biography of the man, William Harvey, with emphasis on the researcher—devotes a generous amount of space to the "Discoverer of the Circulation of the Blood." The final chapter, says the author, relates "Harvey's discoveries to the findings of modern science" . . . "in full accord with the great physician's expressed hope that his work should be reconciled with later discoveries." This is another book well worth reading.

A TEXTBOOK OF HISTOLOGY, Seventh Edition by Alexander A. Maximow, late Professor of Anatomy, University of Chicago, and William Bloom, Professor of Anatomy, University of Chicago, Illinois. W. B. Saunders Company, Philadelphia, Penna. & London, England. 1957. 628 pages. 1082 illustrations, 265 in color, 631 figures. $11.00.

With the increasing interrelation of chemistry and histology, new techniques have been developed. With the solution of some of the problems in histology, others have arisen. Many new illustrations have been added to this section. Electron micrographs have increased the value of this book immensely, and the color illustrations are as beautiful as any we have seen. While it is a "must" in the histology laboratory, this volume is of considerable worth in any clinical laboratory.

CHEMISTRY OF ERYTHROCYTES—Clinical Aspects, by Hans Behrendt, M.D., Associate in Pediatrics, New York Medical College, Metropolitan Medical Center, New York, N. Y., Charles C. Thomas, Publisher, Springfield, Illinois, 1957. 227 pp. 57 tables. 23 charts. $5.75.

A reference volume for the hematologist as well as for the chemist, the first three chapters are devoted to the structure, chemistry, and functional organization of the erythrocytes, and the separation of formed elements of whole blood. Chapter III on Hemoglobin and Its Derivatives is transitional to the chemistry of these cells. Each of the chapters thereafter deals with another important group of the chemical constituents; Proteins and Non Protein Nitrogenous Substances, Carbohydrates, Lipids, Water and Electrolytes, Trace Elements and Enzymes. Although techniques are not included, ample references are given for utilization of the information in this volume.

(Continued on page 300)

DETECTION AND INCIDENCE OF AMEBIASIS IN NORTHERN MISSISSIPPI*

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Introduction

The recognition and differentiation of the intestinal protozoa constitute one of the most important and most difficult phases of animal parasitology. The diagnosis of amebiasis depends upon the demonstration of *Endamoeba histolytica* in material obtained from patients suspected of harboring the parasites. It is most unfortunate from the standpoint of ease of diagnosis that the intestine of man is infested with four other species of amoebae, all non-pathogenic, which require differentiation from the pathogenic *Endamoeba histolytica*.

The number of trophozoites or cysts passed in the feces of a patient with amebiasis varies greatly. One day there may be so many amoebae that a single fecal smear contains more than a thousand, and a day or two later several smears may have to be searched before a single amoeba can be found. Whether the amoebae are numerous or scanty, the probability of detecting and identifying *Endamoeba histolytica* in the feces depends directly upon the skill and interest of the individual worker. In routine examination, a skilled technologist will discover about 75% of *Endamoeba histolytica* infections in a series of three stools, at least one of which would have to be mushy in consistency. Technologists who have not had adequate instruction or who have not had their eyes open to the vagaries of protozoan life discover only the most obvious forms, such as the giant trophozoites of *Endamoeba histolytica* crawling across the microscopic field in a dysenteric fecal discharge. They may examine hundreds of stools without ever seeing an amoebic cyst or trophozoite.

The maintenance of a high degree of accuracy and dependability in the demonstration of *Endamoeba histolytica* is not an easy task and requires not only a skilled technologist but one who is interested and who has patience, for proper microscopic examination of a stool is time-consuming and requires concentrated effort. Competency in this field of diagnostic work is obtained only by careful and lengthy study of specimens containing type forms of the various amoebae and thus acquiring a thorough knowledge of the morphology of each species. The competent technologist will learn to recognize not only typical forms of *Endamoeba histolytica* but will also be able to recognize the atypical or degenerate forms, the constituents of the cellular debris, and the host of other elements found in feces which constitute the diagnostic pitfalls so frequently encountered.

Purpose

Three years ago, at this 150 bed general hospital, an intensification of interest in amebiasis occurred and the method of collection and of examination was standardized with an emphasis on thoroughness. The methods used and results obtained are reported here in order to show others the effects that can be produced by careful examination of stool specimens.

* Parasitology Award, 1957. Read before Annual Convention, Chicago, Ill., June 1957.

Method of Stool Examination

Adult patients referred for routine stool examination receive one ounce of a saline cathartic (Fleet's Phospho-Soda). Children receive an amount of cathartic proportionate to their age. The first three stools following administration of the cathartic are examined. Each stool is collected in a half-pint cardboard container (Dipso Cups) marked with the patient's name and the number of the stool; i.e., whether first, second or third specimen. Each specimen is examined immediately.

Mounts are made on 3 inch x 2 inch glass slides using ⅞ inch, No. 2 thinness cover slips. The 3 x 2 slides are preferred because there is less chance of contaminating the hands and because the large slides simplify the use of a seal (melted one-half vaseline and one-half paraffin) rimming each mount. The seal is applied with a simple water color brush. This seal keeps the mount from drying out and also cuts down on the motion of the field so that the stools are more easily examined. The name of the patient and the number of the specimen is written on each slide with a glass-marking crayon. Two mounts are routinely made on the first specimen, one from the formed portion of the stool and one from the portion of a semi-liquid consistency. One mount is made from each of the other two specimens.

Mounts are prepared by placing a drop of normal saline on the slide and stirring a selected small particle of feces into it with an applicator stick. A cover slip is applied, avoiding the trapping of air bubbles. The film should be thin and uniform so that when seen through the microscope the various fecal components will be separate and clearly illuminated. In thick films amoebae are frequently missed and even when found may be difficult or impossible to identify because they are hidden in the fecal matter. A common method for testing the thickness of a mount is to place the smear over newsprint and if the thinness of the preparation is satisfactory the print will be barely legible. In saline preparations, at least 10 minutes should be allowed for the trophic amoebae to settle down and start their ameboid activities. If examined too soon, all the trophic amoebae will be rounded up.

The wet smear in normal saline solution is intended for the study of amoebae in the living condition. Not all the morphological features from which the identification is made can be seen in the living amoebae, but in spite of this limitation, the normal saline smear is the most important part of the examination. It is the preparation used to determine whether the feces is negative, positive or probably positive and with few exceptions it is the best preparation for this purpose. If the normal saline smears are negative, revealing no suspicious looking objects which might be amoeba, no other preparation is examined.

The nuclei of trophic amoebae are indistinct or entirely invisible in saline preparations. Iodine solution is unsatisfactory for studying trophozoites since it may destroy the entire organism. Due to these difficulties, Svenson (1935) highly recommended a staining solution that was first described by Quensel in 1918 for the study of trophic amoebae in temporary preparations.[1] Suspension of a fecal sample is made in saline and a drop of Quensel's stain mixed with it. This preparation is covered with a cover slip, rimmed with vaseline and paraffin mixture

and allowed to stand for ten to twenty minutes. The trophic amoebae are stained a pale blue with their nuclei a deeper shade. The stained nuclei present the same morphological characteristic as in a permanent hematoxylin preparation. After one to two hours, the organisms become over-stained and can no longer be identified. We do not use this preparation routinely in examining stools but find it quite useful at times as an aid in determining the type of amoeba present.

If amoebic cysts are found, iodine staining may be used to facilitate study of their nuclei and to stain the glycogen masses. A weak iodine solution is preferred for staining the cysts because a high concentration of iodine tends to coagulate the fecal particles and to destroy the refractile nature of the wall of amoebic cysts. There are several iodine solutions that can be used satisfactorily. We prefer Lugol's solution diluted five times. The mount is made as are saline mounts, substituting the iodine solution for saline. The stained preparation is allowed to stand for five minutes before it is examined.

In instances where cysts are few in number or where they cannot be detected in mounts where trophic amoebae are found, we employ a modified zinc sulfate centrifugal flotation concentration of the formed stool. This technique is a modification of the original Faust method.[3] We omit straining the fecal suspension through gauze and wash by centrifugation only once instead of two or three times as recommended in the original method. It gives good results and saves time. A cover slip is placed over the concentration mixture while it is standing and at the end of 10 minutes it is gently removed with a straight upward motion. A drop, containing cysts (if present) will adhere to the underside of the cover slip. This is lowered onto a slide containing a drop of weak iodine solution.

An essential for the detection and study of amoebae is a good microscope and a good microscope light. The condenser must be lowered and the iris diaphragm adjusted to the point where objects in the smear have maximum refractility without glare. The unstained amoebae stand out in this light as refractile shining bodies with a bluish or greenish tint. They can be picked up readily with the low-power objective and this magnification is the most practical for the search. The examiner should make every effort to train himself to use the low-power objective in order to save time. The high-dry objective or the oil immersion lens used with water instead of oil are employed to bring out the morphological features necessary for differentiation. It is wise for the technologist to always check his findings by searching the smear with the high-dry objective since small trophozoites or cysts may be present. In order to cover a smear adequately and to avoid searching the same area more than once, the examination must be made in a systematic and orderly manner using the mechanical stage. The importance of the search cannot be emphasized too strongly since undoubtedly most mistakes in the examination for amoebae are due to failure to find rather than failure to identify.

In the saline mounts, we search for refractile, shining objects under low magnification (16 mm objective). When a suspicious object is seen the high power (4 mm objective) is used to look for differential charac-

teristics in both the trophozoites and cysts. As the mounts are examined the trophozoites are classified as to size, type of motility, character of pseudopodia, endoplasm and its inclusions and the visibility of the nuclei. Cysts are classified as to size, shape, chromatoid matter and the number of nuclei present.

The most common species of amoeba occurring in the intestine of man are *Endamoeba histolytica, Endamoeba coli,* and *Endamoeba nana* and in routine diagnostic work these must be differentiated.

Endamoeba histolytica trophozoites in diarrheal or dysenteric stools are usually a little larger than *Endamoeba coli,* but they are usually a little smaller in non-diarrheal stools. Size, then, is of practically no importance in the differentiation of these two species, but in the case of *Endamoeba nana* is it of importance as this is a very small amoeba and never attains the average size of the other two species. There is a small race of *Endamoeba histolytica* that is similar in size to *Endamoeba nana.*

In freshly voided stools, the trophozoites of *Endamoeba histolytica* exhibit an active, progressive, directional crawl. The entire amoeba appears to advance in a definite direction, pushing its way through fecal material on the slide. There is a rapid succession of ectoplasmic projections. The amoeba becomes elongated and ribbon-like, with an anterior advancing and a posterior dragging end to which granular debris is frequently attached. When this typical motility is seen in small trophozoites, it identifies them as *Endamoeba histolytica* rather than *Endamoeba nana.* The *Endamoeba nana* usually has a billowing, overlapping motion to the pseudopodia with very little progressive motility. In *Endamoeba coli,* motility is almost always comparatively sluggish. The pseudopodia are sluggishly protruded and lazily withdrawn. A definite direction is not maintained for any length of time as in *Endamoeba histolytica.*

The pseudopodia are formed by extrusion of the ectoplasm in all three species and the character of the pseudopodia is of real diagnostic value. The pseudopodia of *Endamoeba histolytica* are finger-shaped or blade-like protrusions from the periphery of the trophozoite and have a clear, glass-like character, which differentiates them from the more granular, greyish endoplasm. The pseudopodia of *Endamoeba coli* are shorter, broader and never long and finger-shaped as in *Endamoeba coli* are shorter, broader and never rapidly and are frequently withdrawn without ensuing motility. The pseudopodia of *Endamoeba nana* are short, broad and blunt and very small in size as compared with *Endamoeba histolytica.* Craig has said that the character of the pseudopodia of *Endamoeba histolytica* in freshly voided stools is so characteristic that when an amoeba with pseudopodia having the above mentioned characteristics, shows rapid progressive motility, one is justified in positively identifying the amoeba as *Endamoeba histolytica.*[3]

The endoplasm of *Endamoeba histolytica* is "clean" and that of *Endamoeba coli* is "dirty" due to bacteria and food debris. The trophozoite of *Endamoeba histolytica* may contain red blood corpuscles and this is a most important diagnostic characteristic. According to Craig,[3] any amoeba that contains red blood corpuscles and is also found in human feces may be considered to be *Endamoeba histolytica.* The endoplasm of *Endamoeba nana* is filled with vacuoles containing bacteria.

The nucleus of an *Endamoeba histolytica* trophozoite is invisible except in the flowing endoplasm during progressive crawl when it may be seen as a

dimly outlined ring. In *Endamoeba coli* trophozoites, the nucleus is almost always visible as a greyish or black ring. The nucleus of *Endamoeba nana* is seldom visible in the living trophozoite.

As stated previously, we sometimes use a mount made with Quensel's stain to make the nucleus more distinct. In examining this mount made with Quensel's stain, we use the oil immersion objective with a drop of water instead of oil. The nucleus of *Endamoeba histolytica* and *Endamoeba coli* are quite similar. The most important and constant differential points are the fine, uniform chromatin granules on the nuclear membrane and the small karyosome in the nucleus of *Endamoeba histolytica* as compared with the large irregular shaped chromatin granules on the nuclear membrane and the large karyosome in the nucleus of *Endamoeba coli.* Frequently, the karyosome in *Endamoeba histolytica* is centrally located, while in *Endamoeba coli* its position is usually eccentric. The *Endamoeba nana* nucleus has a relatively large karyosome and no chromation granules on the nuclear membrane.

To summarize, positive identification of an amoeba as *Endamoeba histolytica* is justified, if an amoeba is found that shows active, progressive motility, has finger or blade-like pseudopodia and contains within its cytoplasm red blood corpuscles.

If cysts of ameobae are found in the smears, we then begin to look for characteristic and differentiating features. Cysts show less variation in size than trophozoites, and the uniformity in size of each species is more obvious. There is overlapping in the size of the cysts so that only a crude grouping as to whether they are small or large is obtained. The *Endamoeba nana* cysts are usually smaller than are those of *Endamoeba histolytica* or *Endamoeba coli,* and the latter are usually spherical. Cysts of *Endamoeba nana* are ovoid or sausage-shaped and this distinctive form serves as an important diagnostic point in their identification.

The chromatoid bodies are highly important in the identification of cysts. The *Endamoeba histolytica* cysts are practically identified by the chromatoid bodies which in the living cysts appear as refractile, glassy, cigar-shaped rods or bars with rounded ends. They are present in about one-half of the cysts.[2] In the small race of *Endamoeba histolytica* these chromatoid bodies are very small but are more brilliantly refractile than in the large race. In *Endamoeba coli* the chromatoid bodies are usually scanty and are seldom seen in more than 10% of the cysts.[2] They appear as spicules or blocks with sharp points. No chromatoid bodies are present in *Endamoebia nana* cysts.

The iodine smear, previously discussed, is employed to show the number of nuclei and their arrangement in the cysts. Cysts of *Endamoeba histolytica* and *Endamoeba nana* contain from 1 to 4 nuclei and the cysts of *Endamoeba coli* contain 1 to 8 nuclei. In all of these species rare cysts may be observed that contain more than the normal number of nuclei, but such findings are exceptional, and cysts with the normal number of nuclei will also be present, so that confusion in the differentiation of species should not occur. The typical immature cyst of *Endamoeba histolytica* is uninucleate and that of *Endamoeba coli* binucleate.[4] The karyosomes of the nuclei are visible with the iodine stain, and as described for trophozoites, those of *Endamoeba histolytica* are usually central and those of *Endamoeba coli* are usually eccentric, whereas the karyosome of *Endamoeba nana* is much larger but its position is of no value in differentiating the species since it can be located centrally or to one side. The nuclear arrangement in *Endamoeba histolytica* is

usually paired and peripheral and quite characteristic. In *Endamoeba coli* a central bunching of the nuclei is observed quite frequently.

In summary, we feel justified in indentifying a cyst as *Endamoeba histolytica* if it contains cytoplasmic chromatin bars with rounded ends or a cigar shape, four paired and peripheral nuclei and centrally located karyosomes in the nuclei.

Results

In our hospital prior to 1954, there was no specified routine for stool examinations. About 50% of the examinations were made on only one stool specimen. The specimen might have been a normally passed stool or it might have been collected following a saline enema or a laxative. Beginning in 1954, we requested that all patients requiring stool examinations have a prescribed dose of Fleet's Phospho-Soda and the first three specimens be sent to the laboratory. At this time, it became our practice to declare as unsatisfactory all specimens that contained any form of oil, barium, bismuth or gall-bladder dye. These substances interfere and are confusing in search for amoebae.

As can be seen from Table I, the percentage of patients having this specified routine for stool examinations has risen from 23 to 85 per cent, and the percentage of stools positive for *Endamoeba histolytica* has risen nine-fold. This increasing percentage of positive stools has created much interest among the medical staff and consequently we have had a corresponding rise in the total number of patients having stool examinations. Although, we still do not have 100% cooperation in our method of collection, we feel that we have made real progress in the past three years, in increasing our findings.

TABLE 1

Results of Stool Examinations for Endamoeba histolytica at Greenwood Leflore Hospital 1953 through 1956

YEAR	No. of Patients Examined	Percent Positive	Percent of Patients 3 Stools Examined
1953	354	1.1	23.4
1954	463	5.6	55.3
1955	510	8.2	59.0
1956	637	9.9	85.4

Summary

Three years ago, at this 150 bed general hospital in the Mississippi Delta region, we standardized our method for the collection and examination of stools for amoebae. Using this standardized method, described herein, we increased positive findings

The percentage of patients having this specified standard stool examination has risen from 23 to 85 per cent, and the percentage of stools positive for *Endamoeba histolytica* has risen nine-folds. This has caused a marked increase in interest in amebiasis among our medical staff and consequently we have had a corresponding rise in the total number of requests for stool examinations.

Our methods and results are reported in order to illustrate the number of cases of amebiasis that might be found as a result of increased care in examining fecal specimens.

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3. Craig, Chas. F.: The Etiology, Diagnosis and Treatment of Amebiasis, 1st edition, Baltimore, Md., The Williams and Wilkins Company, 1944.
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AMONG THE NEW BOOKS

(Continued from page 293)

ANALYTICAL MICROSCOPY—Its Aims and Methods in Relation to Foods, Water, Spices, and Drugs. 2nd Ed., by T. E. Wallis, D.S.C. (London), F.R.I.C., F.P.S., M.I. Biol. Member of the Society for Analytical Chemistry. Little, Brown and Company, Boston Mass. 1957. 215 pp., 90 illustrations. 5 plates. $5.50.

Many practical applications may be made of the material presented in this book. Of primary interest in every laboratory is, of course, the introductory chapter which gives the limitations of the microscope, and discusses the fundamentals for its use. The authors use illustrations generously. As most of the specimens considered will be found only upon rare occasions, the reference value of this book is somewhat limited in the clinical laboratory.

LECTURE NOTES ON THE USE OF THE MICROSCOPE. 2nd Edition, by R. Barer, M.C., M.A., B.Sc., M.B., B.S., University Demonstrator in Anatomy, University of Oxford. Late Johnston, Lawrence and Moseley Research Fellow of the Royal Society. Charles C. Thomas, Publisher, Springfield, Ill., 76 pp. 7 diagrams. $1.50.

A very practical volume that should be in the library of every science or medical laboratory where students are beg'nning to use the microscope. Every user of this basic piece of laboratory equipment should read these lecture notes, which include the physical principles, information on lenses, practical instructions and comments on illumination, and finally the care and cleaning. An appendix includes some simple experiments which will familiarize the microscopist with his equipment.

PERIPHERAL CIRCULATION IN HEALTH AND DISEASE, Walter Redisch, M.D., F.A.C.P., Associate Professor of Clinical Medicine, New York University College of Medicine and Francisco F. Tangco, M.D., B.S., Assistant Professor of Medicine, University of Philippines Medical School, Manila, P. I., Research Fellow, U. Y. U. R. L. de C. H. Saunders, M.D., F.R., S.E. Professor and Head of the Dept. of Anatomy, Dalkonsie University Medical School, Halifax, Nova Scotia, Canada. Greene and Stratton, N. Y. 1957. 15 pp. 31 illustrations (2 color). 4 tables. $7.75.

Part I correlating with Part V, sets the stage for an evaluation of the diseases affecting the peripheral blood flow by the establishment of basic aspects.

Parts II and III give the "Pathologic Alternatives" and "Physiologic Responses" to such disturbances in the blood flow.

These four sections will be of some interest to the researcher in hematology.

Part IV, on Management and Therapy, is entirely for the physician.

For the Medical Technologist who is also X-ray Technician, there is the new book on X-RAY TECHNOLOGY by Charles A. Jacobi, B. Sc., RT(ARXT), MT(ASCP), Head, X-ray Technology, and Donald E. Hagen RT(ARXT), Instructor, X-ray Technology, both of Oregon Technical Institute, Oretech, Oregon. The C. V. Mosby Company, St. Louis, Mo. 1957. 419 pp. 320 illustrations. $9.75.

Also received are:

DORLAND'S ILLUSTRATED MEDICAL DICTIONARY, 23rd Ed., W. B. Saunders Company, Philadelphia and London. 1957. 1598 pp. 50 plates, generously illustrated (more than 700). Tables of Modern Drugs and Dosage, and Fundamentals of Medical Etymology. $12.50.

SCOVILLE'S THE ART OF COMPOUNDING, 9th Ed., by Glen L. Jenkins, Dean and Professor of Pharmaceutical Chemistry, Purdue University School of Pharmacy, Lafayette, Ind., Don E. Francke, Chief Pharmacist, University Hospital, University of Michigan, Ann Arbor, Michigan, Edward A. Brecht, Dean and Professor of Pharmacy, University of North Carolina, Chapel Hill, N. C., and Glen J. Sperandio, Assoc. Professor of Pharmacy, Purdue University School of Pharmacy, Lafayette, Ind., The Blakiston Division, McGraw-Hill Book Co., Inc. New York, Toronto, London. 1957. 551 pp. 43 tables. $11.00.

MODERN APPLIED PHOTOGRAPHY, by G. A. Jones, M.A., A.R.I.C., F.R.P.S., Philosophical Library, Inc., New York, N. Y. 1957. 162 pp, 18 plates, $4.75, which summarizes some of the practical applications of photography to science and technology.

FOOT TROUBLES, by T. T. Stamm, F.R.C.S., Philosophical Library, Inc., New York, N. Y. 1957. 122 pp. 9 plates. 8 plates. $4.75.

THE RARER ANTIBODIES, THEIR DETECTION, IDENTIFICATION AND CLINICAL SIGNIFICANCE

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Reasons for the rarity of certain antibodies

Some of the antibodies to red cell antigens are rare because of one or more of the following reasons:

(1) The antigen has an exceedingly low frequency—the so-called private blood groups, e.g. the Miltenburger antigen.[1]

(2) The antigen has an exceedingly high frequency—the so-called public antigens—and therefore the chances of making an antibody to this antigen are exceedingly low, e.g. U antigen.[2]

(3) Although correctly classified as a red cell antigen, it is of low antigenicity in man and animals, e.g. the Jk^b antigen.[3]

(4) The antigen may be common in certain races but absent in others. Therefore only in areas where donors of mixed races are used, irrespective of the race of the recipient or where mixed marriages occur, will there be chance of immunisation producing these antibodies, e.g. the Vel antigen.[4]

Detection of the rarer antibodies

Detection of these antibodies depends upon:

(1) Having available red cells with the rare antigens. This in turn may depend upon racial differences or similarities between the owner of the antibody and the cells used for testing.

(2) The technique used.

(3) The degree of training of technical staff.

It is impossible for every laboratory carrying out blood group work to have available red cells with all the rare antigens for two reasons:

(a) there is not sufficient of the rarer sera for typing their staff or regular donors, and

(b) with some of the rarer antigens, even if they had sufficient sera, the chances of having sufficient staff to provide a donor with a rare antigen is small.

This in turn means employment of one of the following either

(i) referring unusual reactions to a large central reference laboratory, or

(ii) keeping the red cells with the rare antigen in glycerol citrate mixture at —20° C.[5] or

(iii) receiving supplies of fully typed cells from a reliable large central reference laboratory, e.g., Panocel.

One other source of cells is worthy of mention. In the serological diagnosis of haemolytic disease of the newborn, it is essential to test the mother's serum against her child's, and if possible, her husband's red cells by all techniques. If natural ABO incompatibility exists then it is essential to neutralize the mother's anti-A and/or anti-B before carrying out the test. This procedure will help in the detection of the private or family blood[1] groups.

To some extent the detection of the antibodies to the private blood groups is a matter of chance, having the cells containing the corresponding antigens. However, the detection of the antibodies to the public antigens presents no problems other than identification.

If we now consider what techniques to use each serologist will have his own particular method, but all will agree that no single technique will detect all known antibodies. Possibly the anti-globulin technique, providing the reagent is used at a minimum of two different dilutions, is the most efficient method.* Therefore in the examination of all sera for antibodies, at least two methods, one of which should be the anti-globulin technique, must be used.

However, no matter how many techniques are employed, untrained staff will fail to detect antibodies and so will trained staff unless they are encouraged to refer all anomalous results and participate in the investigation. Lack of interest in the routine work of the laboratory or the personnel performing the work will result in the odd reactions being ignored. Team spirit, enthusiasm for truth and research are essentials for the finding of the rarer antibodies as well as the equipment, material and technical "know-how."

Identification of the rarer antibodies

The rarer antibodies can be classified into two broad categories—those which react with very few cells, e.g. anti-Wright[6] and those which appear to react with all cells, e.g. anti-U.[2]

If the antibody appears to react with only one person's red cells then to identify it the following procedure is essential:

(i) if the reacting serum is from a group O person, exclude that the reacting cells are not a sub-group of A, e.g. A_4.[7]

(ii) Exclude that the cells are not poly-agglutinable by testing with a series of AB neutral[8] serum. Proving that the reacting antigen is an inherited character, an essential part of the investigation, also excludes poly-agglutinability.

(iii) Study the ethnological relationships of the owner of the serum and the owner of the reacting cells. The antigen may be comparatively common in some races and absent from others.[9]

(iv) Test the serum against as many red cells as possible to try and find other examples of the reacting antigen.

(v) Having proved the reaction to be due to an inherited red cell antigen, and having excluded poly-agglutinability, sub-groups of A and ethnological problems, for full identification co-operation with other serologists is essential. This will be dealt with separately.

Antibodies apparently reacting with all cells are not uncommon, but few are examples of antibodies reacting with public antigens. To identify these the following procedure is followed:

(i) exclude the antibody as being a pan-agglutinin by testing the patient's cells against his own serum, which should be negative.

(ii) Exclude the reactions as being due to a mixture of antibodies. In this respect the titration[10] scores against various cells will be indicative of mixtures of antibodies and these can be separated by absorption and elution techniques. Inclusion in the test panel of cells

* Since submitting the manuscript for publication, the author has had the opportunity of studying various commercial anti-human globulin sera available in America, and comparing them with anti-human globulin sera issued in Great Britain. In the latter country the raw serum is issued and the laboratory users dilute it to the required dilution, either for detecting gamma gobulin type antibodies, e.g. Rhesus antibodies, or the non-gamma globulin type antibodies, e.g. anti-Jk^a. In the United States the author found that the anti-globulin sera issued by some firms had been diluted before issue, so that they would only detect gamma globulin type antibodies. For the detection of all types of antibodies that coat red cells, it is necessary that the reagent used will detect more than gamma globulins.

of the same ABO and A sub-type as the donor of the serum are useful for identifying mixtures of anti-Lewis antibodies and anti-H. Several examples of these, active at 37° C, have been observed and while they are active against all group O cells, inclusion of A_1 cells of various Lewis types helps in proving the reactions to be due to a mixture of antibodies.

(iii) The possession of an antibody to a very common antigen indicates an exceedingly rare genotype and therefore a high chance of consanguinity in the parents and the possibility of close blood relatives having the same rare genotype.[11] The parents should be questioned for relationship and the serum tested by titration against the parents, the brothers and sisters of the donor of the serum. Dosage may show the parents to be heterozygotes and there may be sibs who do not react with the serum.

(iv) The final identification of an antibody to a public antigen, as in the case of antibodies to family antigens, depends upon co-operation with other laboratories. This final stage will now be dealt with.

Except in acquired haemolytic anaemia, no individual possesses antibodies to antigens within the red cells. Therefore the testing of cells of the owner of the serum thought to have a rare antibody is an essential part of identification, e.g. if the serum is thought to be anti-Tj^a then the cells of the owner of the serum must not react with known anti-Tj^a. Also in the identification of antibodies to the private blood group antigens, the cells reacting with the antibody must be tested against as many of the sera to this group of antigens as possible. Very few, if any, laboratories exist which possess examples of all known rare anti-sera and examples of red cells containing the corresponding antigens. It is essential therefore in identification of the rarer antibodies to co-operate with other laboratories by exchange of sera and blood samples. Unfortunately supplies of some of the rarer sera do not permit wide distribution and it is essential that reference laboratories be available in each country, if possible, for the complete identification of these antibodies. In this respect W.H.O., or U.N.E.S.C.O., or U.N., organisations may be willing to financially aid such laboratories.

Clinical considerations

As part of the investigation of antibodies to public antigens, people will be found of the rare genotype suitable to be donors for cases possessing these antibodies. These people should be encouraged to act as donors and their willingness to be donors should be known to all transfusion organisations throughout the world. While some may have a good memory for the literature reporting these rare bloods, the setting up of an international donor panel composed of these rare donors is now due.

Finally, these rarer antibodies have been mostly discovered investigating the cause of either a haemolytic transfusion reaction or haemolytic disease of the newborn, i.e. most of the rarer antibodies appear to be of clinical significance. However, the future may show that some of the rarer antibodies may not be of clinical significance in that they do not react at body temperatures. Indeed as we have shown in our own laboratories anti-Wr^a [12] can occur without apparent antigenic stimulus although when Holman[6] first reported this rare antibody it had caused haemolytic disease of the newborn.

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A METHOD FOR MAKING POUR PLATES WITH SPECIAL REFERENCE TO BLOOD CULTURES*

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Blood cultures, their preparation and results, have been the subject of much thought. Considerable ingenuity has been exhibited in the techniques by which they are prepared (see, for example, Marwin, 1949). Many of the published techniques for culturing blood include only fluid media (Conner and Mallery, 1951; Feder, 1937). The omission of the ordinary Petri dish type of pour plate may be justified by the high rate of contamination in such dishes. It is true, also, that the amount of effort and manipulation in drawing and culturing of blood tends to be inversely related to the rate of contamination of blood cultures.

However, the preparation of pour plates is highly desirable procedure if these can be made efficiently. They allow, in many instances, a more rapid identification of any bacteria which may be present. Such plates, when prepared from a measured quantity of patient's blood give some idea of the "intensity of bacteremia" (Topley and Wilson, 1946). In certain instances, bacteria which do not grow well, or not at all, in fluid medium will grow in such plates. If one contaminating bacterium gains entry into a properly poured plate it will result, as a rule, in the formation of only one colony; whereas one bacterium will usually contaminate an entire bottle of fluid medium with the result that a false report of bacteremia may occur, or a true bacteremia may be masked by the presence of a rapidly growing contaminant. Pour plates in Petri dishes are readily contaminated during laboratory examination, as well as during their preparation, and the length of time they can be incubated is sharply limited by this factor as well as by the rapid drying which occurs during incubation. The spectacular appearance of plates poured from bacteremic blood makes them excellent teaching material.

If Petri dishes are replaced by ordinary four ounce ungraduated screw capped medicine bottles, equipped with rubber diaphragm and drilled screw cap, all the advantages of the Petri dish are retained, and many of the disadvantages are avoided.

Methods

In the first nine months of 1954, 477 diagnostic blood cultures made by members of the resident medical staff of the University of Chicago Clinics were examined by the Clinical Microbiology Laboratories of the Department of Medicine. This series included two Petri dish agar pour plates and a bottle of fluid medium.

The change from the Petri dish pour plate to the bottle type of plate was made in October of 1954. The next 477 blood cultures furnished a basis for the comparison between the two techniques. Each culture consisted of the fluid medium bottle and of two agar pour bottles as described below. In each series penicillinase or PABA was added aseptically in the laboratory before the blood was introduced where this was indicated. The fluid medium was a common factor in both series.

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In addition to 50 ml of fluid medium (trypticase soy broth, BBL #01-162) in the McCartney bottle* (Mackie and McCartney, 1953), the use of two agar media for each blood culture is advisable because of differences in ability to support growth and show hemolysis.

Of the two agar media, one was reconstituted blood agar base (Difco #B45); the second was reconstituted trypticase soy agar (BBL #01-168). For the Petri dish pour plates, 18 ml of the agar was sterilized in 1 x 8 inch glass tubes, allowed to gel, and stored in the refrigerator.

Eighteen ml of agar medium was placed in the four ounce medicine bottle, the dimensions of which were about 127 x 50 x 35 mm, and the rubber diaphragm* inserted into the plastic cap which has a four mm hole drilled through its center. The cap was threaded onto the bottle tightly and covered completely by a piece of Kraft paper held in place by a rubber band (Figure 1). All three media were sterilized at 15 pounds of steam pressure for twenty minutes in an automatic recording autoclave. The trypticase soy broth was stored at room temperature. Quantities sufficient to last about one week were prepared in advance. The trypticase soy agar and blood agar base were each prepared in similar quantities, a yellow glass bead being placed in the trypticase soy agar for identification. Every other day a supply of the agar bottles was melted in the autoclave at ten pounds of steam pressure for five minutes. This supply was then placed in the 50C water bath and allowed to come to temperature equilibrium before use.

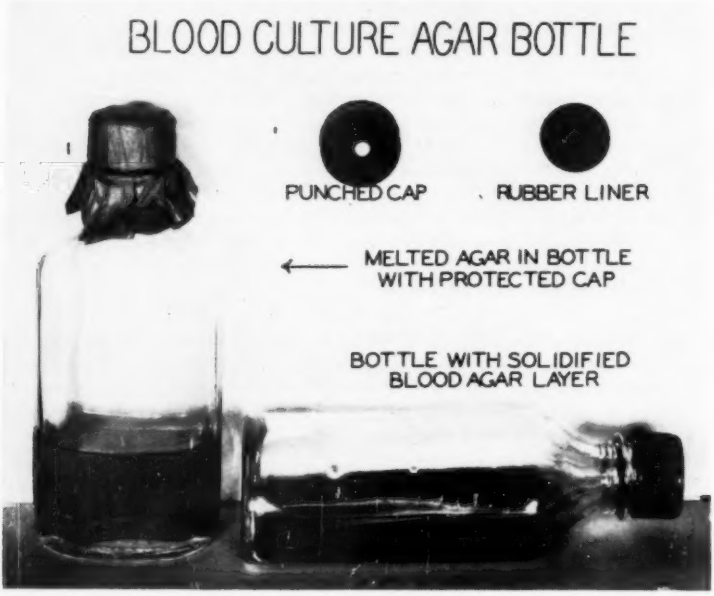


Figure 1.

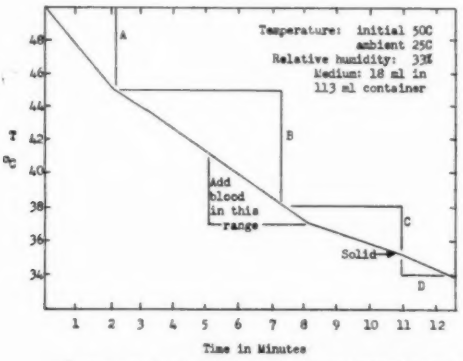
* Obtainable from the Albimi Laboratories, Inc., 16 Clinton Street, Brooklyn 2, New York.

When a blood culture was desired, a member of the resident medical staff, or a medical student under the direction of the resident staff, took the necessary equipment to the bedside of the patient, after which he obtained the media from the laboratory.

For Petri dish pour plates, the agar was heated in the laboratory, to 100C until completely fluid, cooled to 46C, and taken to the patient's room in a vessel containing one liter of water at 46C. One ml of blood was placed in one of the tubes, two ml in the second. It was mixed by rolling the tube between the palms of the hands, and poured into a Petri dish, where it was allowed to solidify before being incubated. Five ml of blood was added to the McCartney bottle by puncturing the diaphragm of the bottle. Aseptic technique was used throughout this process.

For the bottle pour plate series a set of blood culture bottles was obtained from a central point where these were stored. The set consisted of one bottle of trypticase soy broth at room temperature, and one bottle each of trypticase soy agar and blood agar base at 50C. In the patient's room, the rubber bands which held the paper covers on each of the bottles were removed. After the venipuncture was completed aseptically, the paper was removed from the broth bottle. The needle was inserted through the rubber diaphragm while positive control was maintained on the syringe plunger. Five ml of blood was placed in this bottle. One ml of blood was similarly placed in each of the other bottles. These were gently mixed and placed, flat side down, on a flat surface until the medium in them gelled. The broth was shaken to avoid clotting. Not less than five minutes, nor more than eight, must elapse between the removal of the bottles from the water bath and the addition of blood (Figure 2). Bottles are incubated in the upright position to save space.

RATE OF HEAT LOSS AND GELATION OF MEDIUM CONTAINING 1.5% AGAR



Temperature drop in all phases by convection-conduction and radiation; in A increased by evaporation of water from outside of container; in C decreased by heat of fusion of agar.

Figure 2.

Temperature drop is produced by:

A. convection-conduction, and radiation; and by evaporation of water from the outside of container.

B. convection-conduction and radiation.

C. convection-conduction, possibly decreased by heat of fusion of agar.

D. as in B.

For incubation anaerobically or in increased CO_2 tension, the caps may be removed aseptically in the laboratory and replaced with sterile nonabsorbent cotton plugs. This is not essential since a good anaerobic atmosphere, or an increased CO_2 tension is obtained, without contaminating the cultures if the caps are loosened one-half turn before placing in the anaerobic jar or the CO_2 jar, where an evacuation-replacement system is used. Where hydrogen is used for producing anaerobiasis, it is essential that the containers be free from hydrogen when they are flamed on opening.

Where brucellosis is considered to be a possibility, trypticase soy broth in 50 ml quantities containing 1% sodium citrate is used, and the cultures are incubated in an atmosphere containing 76 mm partial pressure of CO_2 (10%). Citrate is not used under any other circumstances.

For picking colonies from the agar bottles, a heavy wire, 22 gauge, needle is used. The tip is bent at about 60 degrees, with the bent part about one cm long, so that it will reach through the entire depth of the agar without the remainder of the needle or the handle touching the agar surface.

Results

The agar stored in bottles at 50C for three days still supported the growth of fastidious organisms. The pH of the media was determined immediately after autoclaving and again after three days at 50C, using a Beckman H-2 pH meter and glass electrode (Table 1). Determinations were made in triplicate on bottles selected at random from the same batch of medium.

TABLE 1
Stability of pH During Storage of Media

Medium	pH after Autoclaving	pH after Storage	STORAGE	
			Time	Temperature
Trypticase soy broth	7.26	7.26	1 week	room
Trypticase soy agar	7.25	7.25,	3 days	50C
Blood agar base	6.87	6.9	3 days	50C

Figure 2 shows one example of the rate of cooling of the melted agar in bottles. A Weston dial type metal stem thermometer was pushed through the cap with sufficient pressure to tear the rubber diaphragm out of place and to allow the thermometer to reach the bottom of the bottle of melted agar which was in the 50C water bath. After the thermometer had reached temperature equilibrium, the bottle with the thermometer was removed from the bath and placed on a wooden laboratory bench. The ambient temperature and the wet bulb temperature were recorded three minutes after removal of the bottle from the bath. The temperature of the medium was noted at thirty second intervals The bottle was shaken once every minute until the agar was solidified. The results of the thirty second readings were plotted against time to yield the curve in Figure 2, with ambient temperature at 25 C and humidity 33%. Another curve obtained by a similar process when the ambient temperature was 33C and the relative humidity was 68% showed, that, under these circumstances, the agar was firmly gelled in twelve minutes.

Cooling rates varied with temperature and relative humidity. Solidifying time, when ambient temperature was in the range 21C to 33C, and relative humidity 33% to 68%, varied from 10.5 to 12 minutes, so that if blood is added between five and eight minutes after removal (without drying) from the water bath, temperature of the medium is never above 43C nor below 37C. It may be noted that, experimentally, agar in a concentration of 1.5% does not have a sharp gel temperature, but gels over a range of about 35.5C to 37C. This is only the apparent gel temperature, under the specified conditions, because if the agar is kept at 37C it gels firmly, but more slowly. Trypticase soy agar gels at a slightly higher temperature than does the blood agar base.

Of the 477 cultures (Table 2) taken in the Petri plate series, the agar medium was omitted, or was not usable due to faulty technique or contamination in 129 or 27% of the cultures. Analogous figures in the bottle series were 34 or 7.1% of the cultures. The total number of cultures judged to be positive in the Petri plate series was 51 or 10.7%, while the number in the bottle series was 50 or 10.5%. In 11 or 2.9% of the positive Petri plate series, agar was negative, omitted, or unsatisfactory, while this was true in 4 or 0.8% of the positive bottle plate series. Table 2 summarizes the similarities and differences of the two series of blood cultures. Table 3 lists the organisms which were found in positive blood cultures of both series.

TABLE 2
Comparison of Results Using Agar Plates and Agar Bottles in Blood Cultures for Diagnostic Purposes

Condition of Culture	PLATE SERIES			BOTTLE SERIES		
	Number	Percentages		Number	Percentages	
Total blood cultures	477	100	....	477	100	....
Agar medium omitted	81	17.0	....	9	1.9	....
Total Positive Cultures	51	10.7	....	50	10.5	....
Broth positive, agar negative or unusable (contaminated or defective)	16	3.3	....	11	2.3	....
Total cultures with agar media	396	....	100	468	....	100
Defective agar						
Usable, one or both	80	....	20.2	114	....	24.4
Unusable, one or both	20	....	5.0	18	....	3.8
Contaminated cultures						
Broth, first examination	31*	6.5	....	23	4.8	....
Agar, one or both, usable						
Plates held up to 5 days	80	....	20.2	....	....	....
Bottles held up to 14 days	....	....	....	7	....	1.5
Agar, one or both, unusable	28	....	7.1	7	....	1.5

* Fourteen broths were contaminated with molds from August 20-29, 1954.

Discussion

Anaerobic or microaerophilic *Clostridia* grow well in the depths of the fluid medium without altering its atmosphere, and aerobic *Pseudomonas* will grow on the surface of gelled medium and in the fluid medium under similar conditions. The gram negative enteric bacteria generally grow well on all the media used, as do most species of micrococci. *Hemophilus influenzae* may be recovered from the trypticase soy broth by subculturing on trypticase soy chocolate blood agar incubated at an increased partial pressure of CO_2, or by subculturing on blood agar in the presence of *Micrococcus pyogenes*, var. *aureus*.

TABLE 3

Organisms Isolated from Positive Blood Cultures

ORGANISMS	Plate Series	Bottle Series
Micrococcus pyogenes, var. **aureus**	12	24
Micrococcus pyogenes, var. **albus**	1	2
Micrococcus species	8	2
Alpha hemolytic streptococci	15	6
Beta hemolytic streptococci	2	2
Streptococcus fecalis	3	3
Diplococcus pneumoniae	1	3
Escherichia intermedium	..	1
Aerobacter aerogenes	1	..
Paracolobactrum coliforme	2	..
Pseudomonas aeruginosa	1	2

In addition, **E. coli, E. freundii, Proteus mirabilis,** and **Pseudomonas** species were recovered from post mortem blood cultures.

Pneumococci and the so called alpha hemolytic streptococci produce the usual zones of alpha type hemolysis. Hemolytic coliforms in the depths of the medium may, on superficial gross examination, be mistaken for beta hemolytic streptococci. A gram stain from the colony will correct this error. *M. pyogenes* var. *aureus* may not produce lysis in the blood if the colonies are deep.

In the absence of sodium citrate, beta hemolytic streptococci (Lancefield group A) will produce lysis of the erythrocytes in a poured plate when lysis is not apparent on the surface of such cultures, presumably in those cases where antistreptolysin "O," but no antistreptolysin "S," is produced. Of 20 strains of beta hemolytic streptococci (Lancefield group A) subcultured in 1% citrate broth, five (20%) failed to grow, but grew well when subcultured to the same medium in the absence of citrate.

Alcohol lamps or other flames are not used in the process of distribution of the blood from the syringe to the bottles. The flame of an ordinary wick type of alcohol lamp is not sufficiently hot to be of any value, and portable flames, when used about the person or beds of patients represent a serious fire hazard. A Weston metal stem dial type thermometer held so that the thermosensitive element is immediately above the flame of a wick type (nonpressure) alcohol lamp for fifteen seconds reached, experimentally, a temperature of 60C. The finger may be passed slowly through such a flame without discomfort.

Irregularities in technique resulted in some agar plates or bottles which were chocolated, poorly mixed, contained lumps of agar or too much or too little blood or many bubbles. These were considered defective, though they did not usually prevent an adequate examination of the plates. In addition, pour bottles in which the agar slipped to the bottom were considered defective. This condition may result from placing the bottle upright too soon, or from improper reconstitution of the dehydrated medium.

The literature does not contain any satisfactory definition of "contamination" with respect to blood cultures. This condition remains one in which each case must be judged on its own merits, by standards which are sometimes subjective. Conner and Mallery (1951) considered that certain growths, on clinical and laboratory evidence, represented

contamination. Such a standard, admittedly unsatisfactory, is used in this study for want of a more objective one.

Organisms considered contaminants in particular instances include micrococci; molds such as *Alternaria* species, *Fusarium* species, *Hormodendrum* species, and *Penicillium* species; diphtheroids; *Pseudomonas* species; and *Bacillus* species. Where contamination apparently occurred in the fluid medium after the first transfer, it was assumed to be laboratory contamination. It was not usually possible to distinguish between laboratory contamination, and contamination during initial preparation of the culture where Petri dish plates were used. Since the pour bottles are not opened for examination unless growth has occurred, laboratory contamination is virtually never encountered.

The number of positive cultures (Tables 2 and 3) in the two series was almost identical. This is largely coincidental; although it may reflect a decrease in number of positive results from cases of subacute bacterial endocarditis, with compensating increase in bacteremias of staphylococcal origin. The number of broth cultures contaminated is essentially the same in both series, if the episode of August 20-29, 1954 (footnote, Table 2), is considered as a special circumstance. Agar medium was omitted from 8.9 times as many cultures in the Petri plate series as it was in the bottle series. This appears to be a reflection of the relative ease and simplicity of the bottle method of making plates. Unusable defective agar occurred with about the same frequency in each series, 5.0% in the Petri dish series, and 3.8% in the bottle series. Contaminated agar plates occurred 9.4 times as frequently in the agar plates as it did in the agar bottles.

Summary

A simple method for the preparation of pour plates in medicine bottles is presented. The effect of the use of the method on the results of 477 blood cultures, compared to the same number of cultures made when Petri dishes were used, is discussed, and the results are found to favor the use of the method.

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THE RELATIONSHIP OF SERUM CHOLESTEROL TO BLOOD LIPID PATTERNS*

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Blood lipids are assuming a progressively important diagnostic and therapeutic role in medicine today. While interest in the blood-lipid patterns has been particularly prominent in the field of atherosclerosis, it has also received a great deal of attention in other realms of medical investigations.[1,2,3,4] It is natural that since determinations of the total elements of blood lipids have been extremely difficult, from a practical laboratory standpoint, a search should be made for a single lipid determination that might reflect the entire lipid patterns.[5] These lipid patterns are defined, in this instance, as cholesterol, phospholipids, the related athero index and alpha and beta lipoproteins. The total blood lipids, total "Fats," have been excluded. One finds in the literature, statements that blood cholesterol is the most practical and inexpensive method of determining all of the blood-lipid elements. It is the purpose of this study to establish the fact that blood cholesterol is not a reflection of the blood-lipid elements. This is not an attempt to relate the blood-lipids to any specific condition, such as atherosclerosis.

Fifty patients were chosen at random from a group analyzed by the coronary profile system.[6] These patients were all male, and represented an age group from 25 to 79. Thirteen of the patients in the group had proven myocardial infarction, and the rest evaluated by standard means and the profile system, were designated as normal. The myocardial infarct patients were included to insure the fact that patients in this study represented not only normal, but also abnormal lipid patterns. For each patient, the total cholesterol was done by the Zak method,[7] phospholipids by using the procedure described by Youngburg,[8] and the athero-index as originally reported by Barr.[9] Beta lipoproteins were determined, using the electrophoretic technic of Durrum.[10] The absolute variation from normal was then calculated, and the per cent variation was then recorded for each of these four values (Figure 1). Alpha lipoproteins were not recorded, as their variation was inversely proportional to the beta lipoproteins.

It is important to describe the selection of normal values for this study, as the mutual normal values determined by the world literature for any given blood lipid, are virtually non-existent. Any textbook or article published, whether foreign or domestic in origin, usually gives a different absolute normal value for each of the lipid elements, and, even more confusing, varies the ranges of normal. It was, therefore, decided, in an attempt to establish normal, and consequently, to establish the absolute and precentile variation from this normal, to scan the more significant publications, and to correlate their normals with the findings of normal in our own laboratory. Using this approach our normal values for cholesterol were 214 mgs % (Figure 2), phospholipids, 224 mgs % (Figure 3), and athero-index 1.19 (Figure 4). As regards the athero-index, correlating the work of various authors substantiates the fact that an athero-index of 1.19 appears to be a recognized normal

* 1st Chemistry S.P.F. Award, 1957.
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PATIENT	Age	★	CHOLESTROL (Normal 214) Mg. %	Deviation Abs.	Deviation Percent	PHOSPHOLIPIDS (Normal 224) Mg. %	Deviation Abs.	Deviation Percent	ATHERO-INDEX (Normal 1.19) PL/C	Deviation Abs.	Deviation Percent	LIPOPROTEINS (Normal 85%) Beta Percent	Deviation Abs.	Deviation Percent
1	68	★	216	2	1	219.	5	— 2	1.1	— .09	— 8	89	4	5
2	53		233	19	9	208.	—16	— 7	1.06	— .13	—11	87	2	2
3	56	★	360	146	68	243.4	—19	8	0.57	— .62	—51	92	7	8
4	60	★	260	46	21	228.8	5	2	0.88	— .31	—26	75	—10	—12
5	32		230	16	7	208.	—16	— 7	0.9	— .29	—25	67	—18	—21
6	60	★	310	96	45	223.6	0	0	0.72	— .47	—39	90	5	6
7	54		385	171	80	233.6	10	4	0.6	— .59	—49	86	1	1
8	53		441	227	106	405.6	182	81	0.9	— .29	—25	80	— 5	— 6
9	60		231	17	8	395.2	171	76	1.7	+ .51	43	89	4	5
10	32		170	—44	—21	278.2	54	24	1.6	+ .41	35	67	—18	—21
11	60	★	180	—34	—16	266.5	43	19	0.95	— .24	—20	92	7	8
12	36		148	—66	—31	215.8	— 8	— 4	1.4	+ .21	18	81	— 4	— 5
13	48		220	6	3	273.	49	22	1.2	+ .01	1	72	—13	—15
14	38		170	—44	—21	201.5	—22	—10	1.1	— .09	— 8	74	—11	—13
15	34		315	101	47	309.4	85	37	0.98	.21	18	92	7	8
16	79	★	320	106	49	598.	374	167	1.8	+ .61	51	91	6	7
17	29		185	—29	—14	364.	140	62	1.96	+ .77	64	74	—11	—13
18	45		140	—74	—35	206.	—18	— 8	1.4	+ .21	18	67	—18	—21
19	53		240	26	12	457.6	234	104	1.9	+ .71	60	86	1	1
20	53		260	46	21	275.6	52	23	1.06	— .13	—11	79	— 6	— 7
21	49	★	240	26	12	260.	36	16	1.08	— .11	— 9	82	— 3	— 4
22	42		244.8	31	14	205.4	—19	— 8	0.84	— .35	—30	94	9	11
23	28		203	—11	— 5	210.6	—13.4	— 6	1.04	— .15	—13	74	—11	—13
24	36		224	10	5	208.	—16	— 7	0.93	— .26	—22	91	6	7
25	54		205	— 9	— 4	242.	18	8	1.09	— .1	— 8	83	— 2	— 2
26	60	★	298	84	39	260.	36	16	0.87	— .32	—27	90	5	6
27	39		208	— 6	— 3	182.	—42	—19	0.87	— .32	—27	84	— 1	— 1
28	41		192	—22	—10	210.	—14	— 6	1.09	— .1	— 8	81	— 4	— 5
29	42		274	60	28	209.	—15	— 7	0.84	— .35	—29	72	—13	—15
30	50		290	76	36	236.6	13	6	0.815	— .37	—31	82	— 3	— 4
31	51	★	279	65	30	235.	11	5	0.84	— .35	—29	93	+ 8	9
32	48		221	7	3	156.	—68	—30	0.72	— .47	—40	91	6	7
33	48		182	—32	—15	192.	—32	—15	1.04	— .15	—13	92	7	8
34	60	★	380	166	78	327.6	104	46	0.86	— .33	—28	89	4	5
35	51		300	86	40	304.	80	36	1.0	— .19	—16	82	— 3	— 4
36	44		190	—24	—11	275.6	52	21	1.45	+ .25	21	77	— 8	— 9
37	66	★	180	—34	—16	210.6	—13	— 6	1.16	— .03	— 2	78	— 7	— 8
38	64	★	184	—30	—14	327.6	104	46	1.78	+ .59	50	85	0	0
39	51		204	—10	— 5	268.	44	19	1.3	+ .11	9	90	5	6
40	56	★	166	—48	—22	468.	244	109	2.8	+1.61	135	89	4	5
41	42		190	—24	—11	286.	62	28	1.5	+ .31	26	87	2	2
42	40		176	—38	—18	304.2	80	36	1.7	+ .51	43	75	—10	—12
43	32		145	—69	—32	218.4	— 6	— 3	1.5	+ .31	26	69	—16	—19
44	26		200	—14	— 7	229.	5	2	1.14	— .05	— 4	79	— 6	— 7
45	31		230	16	8	260.	36	16	1.13	— .06	— 5	79	— 6	— 7
46	25		180	34	16	219.	— 5	— 2	1.2	+ .01	1	89	4	5
47	27		175	39	18	229.	5	2	1.3	+ .11	9	86	1	1
48	25		205	— 9	— 4	211.	—13	— 6	1.0	— .19	—15	83	— 2	— 2
49	22		158	—56	—26	219.	— 5	— 2	1.38	+ .19	15	85	0	0
50	33		214	0	0	228.8	5	2	1.07	— .12	—10	87	2	2

* Male patients, age group 25 to 79. ★ Post Myocardial Infarction patients.

Figure 2—Normal values for blood cholesterol.

AUTHOR	Year	No.	m	Age in Years	f	Age in Years	Tch			Fch			Percentage		
							Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
Bloor & Knudson	1917	26											30	54	40
			17				180	291							
					9		140	247							
Gardner & Gainsborough	1927	43	22	19—58			100	223	169						
					21	17—25	78	208	153						
Page et al.	1935	67	67				109	376	232				22	72	37
Sperry	1936	91					132	392	208				24	30	27
Brochiner-Mortensen & Moller	1940	20	10	19—53			147	309	228						
					10	19—53	178	299	221						
Peters & Man	1943	174		10—68		10—68	107	320	194				24	32	28
Dahlberg & Josephson	1948	25	25	20—30					219						
		27	27	60—65					250						
		25			25	20—30			233						
Auken	1948	101	51	17—43	50	19—41	137	302	200				17	36	26
Keys	1949	541	541	17—74					219						
Kornerup	1950	117	80	19—96			142	320	214	29	97	56	17	33	26
						19—39									
					37		154	318	229	28	86	60	18	31	26
						61—96									
Gertler et al.	1950	146	146	20—59			148	332	225						
Goldbloom	1952	15	6	22—72	9	22—72			197	60	90	76			39
Katz et al.	1953	30	17	33			122	281	202						
				(mean)	13	36	166	239	200						
						(mean)									
Swahn	1953	10	6	14—57	4	10—57	152	275							
Keys et al. (Neapel)	1954	83	83	20—55					221						
Keys et al. (Minneapolis, St. Paul)	1954	59	59	50—60					280						
Gertler & Oppenheimer	1954	44	22	18—35					197						
					22	18—35			187						
		50	25	65—86					226						
					25	65—86			282						
		26	26	25—50					225						
Present investigation	1956	195	102	20—91			114	238	175	43	92	66	30	49	37.5
					93	20—91	131	241	182	43	99	68	25	47	37.2

Tch: Total cholesterol (mg/100 ml). Fch: Free cholesterol (mg/100 ml). p: Percentage of free cholesterol in total cholesterol.

Figure 3—Normal values for phospholipids.

AUTHOR	Year	Number	m	Age in Years	f	Age in Years	PHL Min.	PHL Max.	PHL Mean
Schreiner and Bilger	1932	....	....	.....	..	.....	290	317	....
Boyd	1933	8	....	.....	8	"young"	....	....	196
Page et al.	1935	66	66	.....	..	.....	....	....	181
Peters and Man	1943	108	....	10—68	..	10—68	153	363	230
Kornerup	1950	104	73	19—96	..	.....	105	280	173
	....	....	....	.....	31	19—96	55	257	195
Ahrens Jr.	1950	20	....	21—38	..	21—38	....	....	219
Goldbloom	1952	15	6	22—72	9	22—72	195	242	218
Petersen	1952	48	21	20—55	..	.....	153	263	208
					27	17—52	175	289	230
Swahn	1953	10	6	14—57	4	10—57	180	255	....
Gertler and Oppenheimer	1954	44	22	18—35	..	.....	....	....	193
	....	....	....	.....	22	18—35	....	....	228
	....	50	25	65—86	..	.....	....	....	258
					25	65—86			258
		26	26	25—50	..	.....			300
Present Investigation	1956	195	102	20—91	..	.....	130	336	245
					93	20—91	161	403	252

PHL: Phospholipids (mg/100 ml).

Figure 4—Normal values after Gertler & Oppenheimer (1954) for athero-index.

	Age in Years	Number	PHL	Tch	Q
m	18—35	22	193	197	0.98
f	18—35	22	228	187	1.22
m	65—86	25	258	226	1.14
f	65—86	25	258	282	0.91
m	25—50	26	300	225	1.33

mean.[11,12,13] In determination of normals for lipoproteins, by using electrophoresis, it has become evident that each laboratory must establish its own normal values according to the technics involved.[14,15,16] A determination of lipoproteins by electrophoresis is an extremely sensitive procedure, subject to innumerable variations such as changes in voltage, heat, drying time of the strips, composition of the buffer and staining solution, as well as the running time for the serum samples.[17,18] Selection of 85% normal for the beta lipoproteins for males, both atherogenic and healthy individuals, has been based on three years study in our own laboratory with our own technics.

Results of these studies, as indicated from the following charts, show that cholesterol is not a reflection of blood lipid elements. One can see that the graphs are not superimposable, and that where cholesterol may show a particular mean deviation (Figure 5), phospholipids (Figure 6), the athero-index (Figure 7), and the lipoproteins (Figure 8), do not show the same variation, nor is there even a semi-quantitative correlation. This can best be summarized by a block chart (Figure 9), which shows the mean deviation of cholesterol as opposed to phospholipids, athero-index and lipoproteins. There may be some question as to the total mean deviation, even if the individual percentile deviations are not the same, but in this case the mean deviation is significant in that the superimposable graphs are not even remotely related. It is also apparent from this study that no one blood-lipid pattern is expressed by the cholesterol, phospholipid, athero-index or the lipoprotein.[19,20,21,22] The assumption cannot be made that because one element is either increased or de-

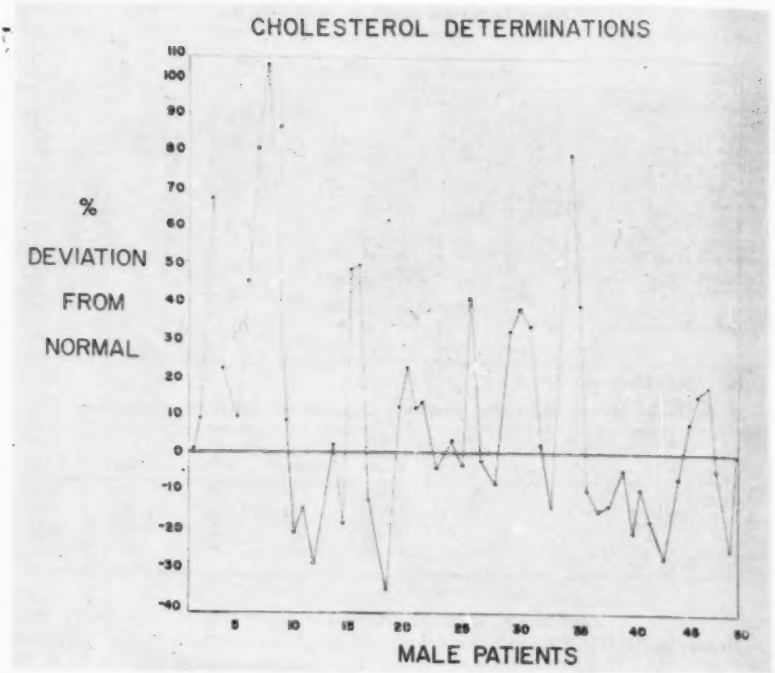

Figure 5—Percentile deviation of cholesterol from normal.

creased, that the others, of necessity, shall show the same deviation.

From this evidence it is concluded that cholesterol in itself does not reflect the rest of the lipid element patterns, the total blood-fat determination being excluded. It is also obvious that the rest of the lipid elements, such as phospholipids, athero-index or lipoproteins also do not reflect the lipid patterns. While this study has been confined to males, it is apparent, from our work, that females do not differ in this respect, and this includes both normal females and those who have suffered from atherogenic disease.[20] Therefore, it is not justified at the present time to select the blood-cholesterol as a reflection of the lipid patterns.

Summary

1) Lipid patterns, as defined by the determination of cholesterol, phospholipids, athero-index and beta lipo-proteins, were determined on fifty male individuals, ages 25 to 79, both normal and proven myocardial infarctions.

2) Normal values for the lipid elements were selected from world literature and our own laboratory experience.

3) Absolute deviation and percentile deviation from normal were then

determined and plotted on graph scales.

4) Results showed that percentile deviation of cholesterol from normal was not reflected in other blood-lipid elements, and blood cholesterol cannot be used as an indicator of other lipid element patterns of a given individual.

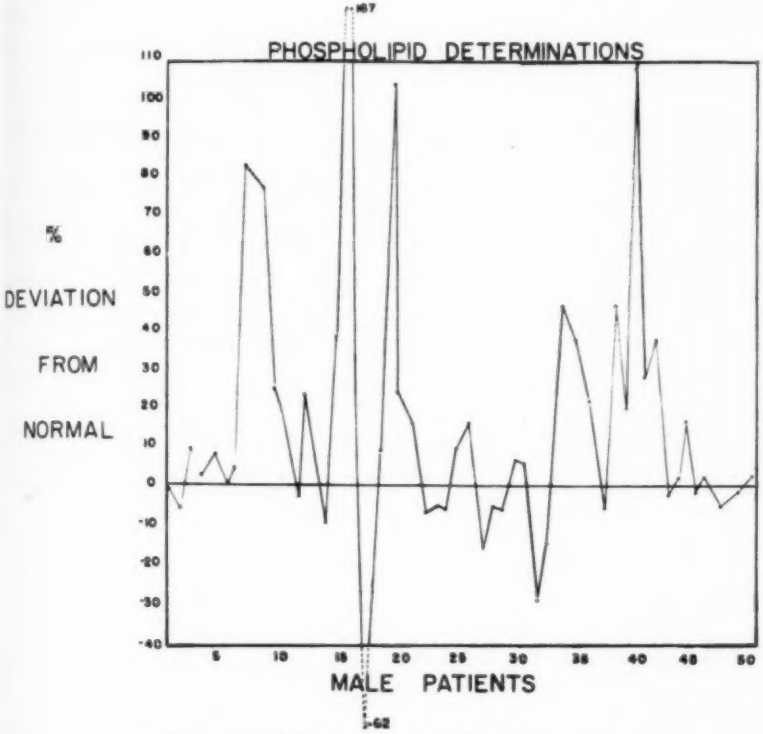

Figure 6—Percentile deviation of phospholipids from normal.

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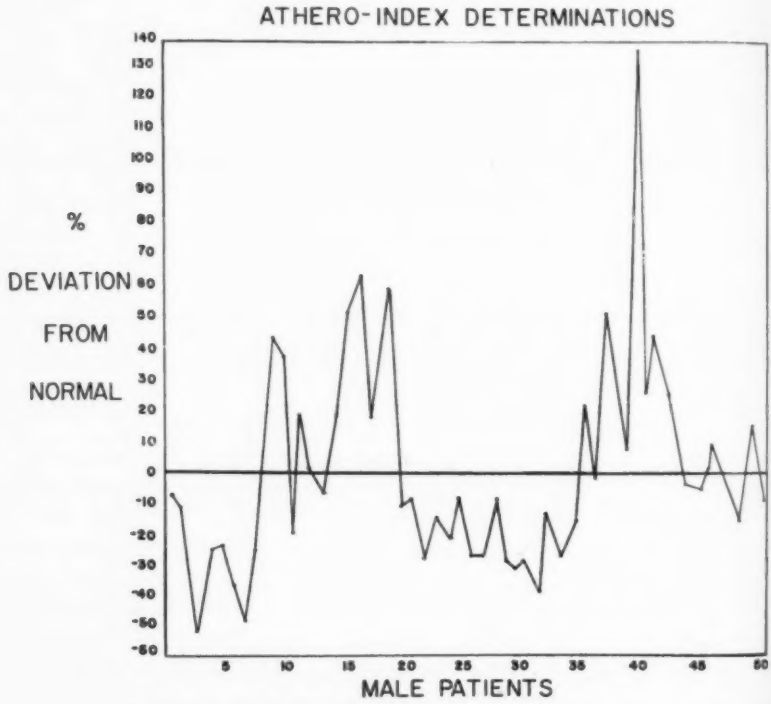

Figure 7—Percentile deviation of athero-index from normal.

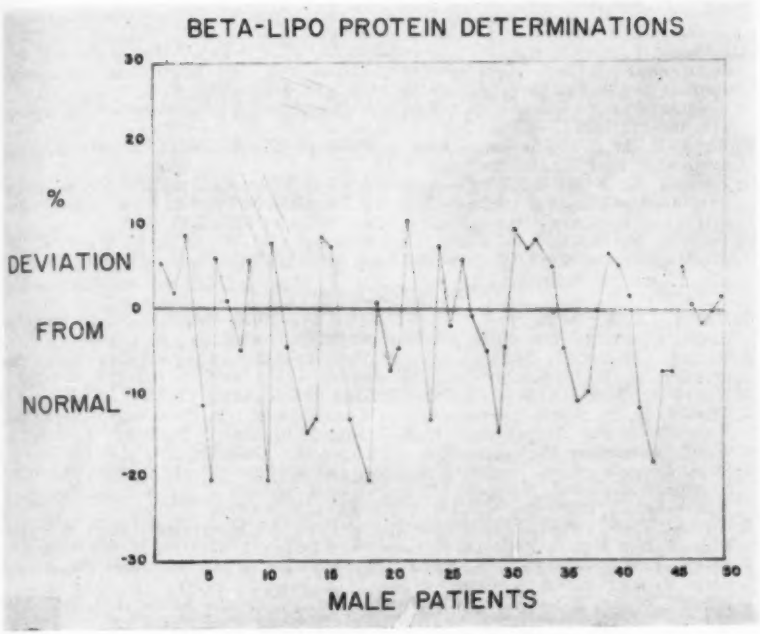

Figure 8—Percentile deviation of lipoproteins from normal.

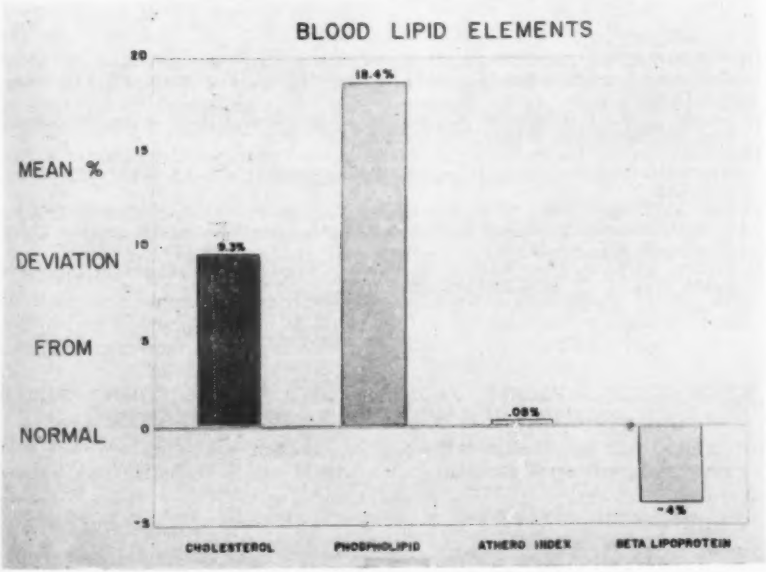

Figure 9—Mean percentile deviation of blood-lipid elements.

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